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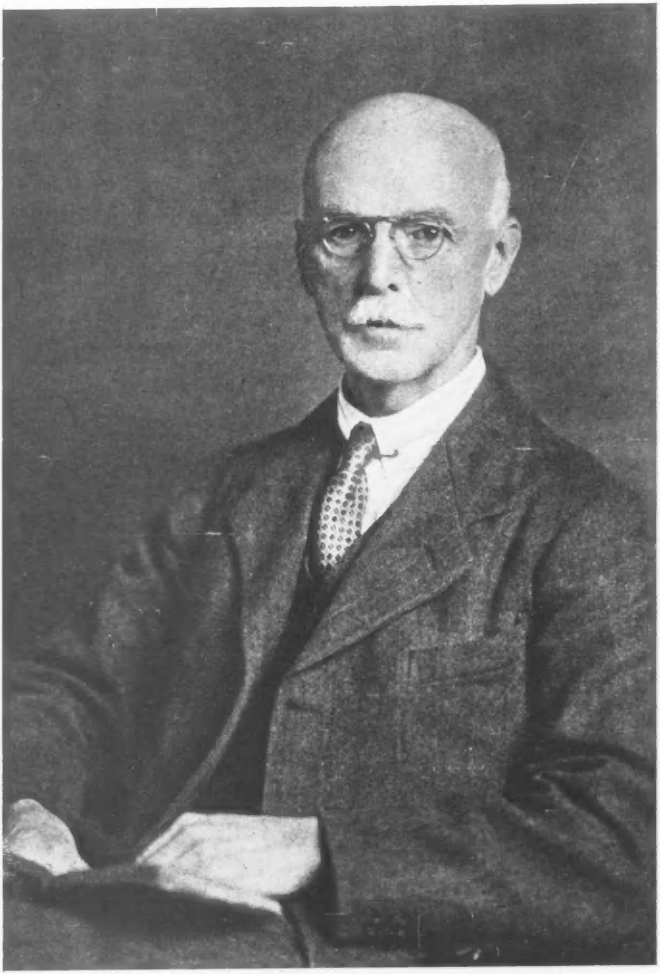

A. HARDEN
(1865-1940)

OBITUARY NOTICES

ARTHUR HARDEN

(1865–1940)

SIR ARTHUR HARDEN, founder-member of the Biochemical Society and for 25 years editor of its *Journal*, died on 17 June 1940 at his home at Bourne End.

Arthur Harden was born in Manchester on 12 October 1865. He was the only son of Albert Tyas Harden, a Manchester business man who had married Miss Eliza MacAlister of Paisley. The family, consisting of Arthur and his sisters, was brought up in a somewhat austere nonconformist atmosphere, like the Scottish Presbyterians, abjuring the theatre and regarding Christmas almost as a pagan festival. At the age of 7 years Arthur was sent to a private school kept by Dr Ernest Adam in Victoria Park, and four years later in 1877 he went on to the Tettenhall College in Staffordshire where he stayed until he was 16, waiting an extra term in order to qualify for the London matriculation. In January 1882, he entered Owens College, Manchester, and studied chemistry under Prof. Roscoe, then at the height of his fame as a teacher. In 1885 he graduated in the Victoria University with first class honours in chemistry and a year later was awarded the Dalton scholarship. It was J. B. Cohen, to whose stimulating teaching Harden doubtless owed much, who suggested the subject of Harden's first research, 'The action of silicon tetrachloride on aromatic amide-compounds', and in the following year the results of this investigation were published in the *Transactions of the Chemical Society* [1886]. From Manchester he proceeded to Erlangen, and under the direction of Otto Fischer prepared β-nitroso-α-naphthylamine and investigated its properties. Having been awarded the degree of Ph.D. he returned to Manchester and there became junior and later senior lecturer and demonstrator under Prof. H. B. Dixon who had succeeded Sir Henry Roscoe as Professor of Chemistry. During part of this time Harden shared with (Sir Philip) Hartog the laboratory teaching, at first in the large qualitative and then in the quantitative laboratory, and for some years he lectured to the Honours students on the history of chemistry, a subject in which he was greatly interested. With characteristic thoroughness he traced out members of the Dalton family and obtained from them information about Dalton's family and procured a photograph of Dalton's birthplace. This interest was shared by Roscoe: together Harden and he studied the note-books and arrived at the conclusion that it was Dalton's investigation of the relations governing the diffusion of gases that led him to formulate his atomic principles; these views were published in two papers on the genesis of Dalton's Atomic Theory [*Phil. Mag.* 1897, p. 153; *Z. phys. Chem.* 1897, **22**, 243], and were also expanded in a book entitled *A New View of the Genesis of Dalton's Atomic Theory*. Harden's interest in Dalton remained, and in 1915, with H. F. Coward, he communicated to the Manchester Literary and Philosophical Society a description of some of the lecture sheets with which Dalton used to illustrate his lectures and which had recently been discovered in the rooms of the Society.

In 1895, Roscoe, stimulated by an article on the teaching of science contributed by H. E. Armstrong to *Nature*, suggested that Harden should collaborate with him in a book for advanced students, and the outcome of this was a text-book to fulfil the needs of students for the higher South Kensington Examination.

In collaboration with F. C. Garrett, a book on *Practical Organic Chemistry* was written, planned to accompany Perkins's *Organic Chemistry*. At Roscoe's request, Harden also undertook the work of revising and editing a new edition of Roscoe and Schorlemmer's *Treatise on Inorganic Chemistry*; this was carried out in collaboration with Colman, a fellow-student who remained throughout one of Harden's closest friends, and the experience must have served as valuable training for the editorial work which was to occupy so much time in his later life. As at this time the salary of a senior lecturer in chemistry at Owens College was only £150 a year, the money obtained by his literary work must have formed a useful addition to the salary.

During these nine years of lecturing and teaching in Manchester, Harden seems to have been chiefly interested in his teaching and literary work. He believed that teaching was the primary duty of a demonstrator and that it was unfair to grumble because the student had not already learnt what the demonstrator was paid to teach him. The Department was then supplying a steady stream of chemists for the expanding industries of the north, and Harden made it his business to become acquainted with their technical problems and often advised firms who had met with some difficulty or other. He published only two papers based on experimental work; both appeared in the *Transactions of the Manchester Literary and Philosophical Society*, one with W. Haldane Gee [1891] on a new form of stereometer, and one on the composition of some iron and bronze implements which had been found by Prof. Flinders Petrie, then Professor of Geology at Manchester [1897]. Harden obtained the consent of Prof. Otto Fischer to continue the work on nitroso-compounds, but nothing further appeared on this subject. He began experiments on the combination of carbon monoxide and chlorine, with a view to studying the phenomenon of photochemical induction, a subject in which Dixon was especially interested; this work was carried out in collaboration with Dyson, but the results were not published until 1903, six years after Harden had left Manchester. It seems therefore that up to the time he left Manchester at the age of 32, Harden had given no indication of any marked interest in scientific research. He had applied for the posts of Principal at the Wandsworth Technical Institute and of Inspector under the Science and Arts Department at South Kensington: fortunately, both these applications were refused, the former on the ground that his training was too highly specialized and the latter on the ground of his youth.

In 1897, Sir Henry Roscoe was treasurer of the British Institute of Preventive Medicine, which had laboratories in Great Russell Street. It was an Institute which fulfilled various functions. It held classes for students of Public Health in bacteriology and chemistry, carried out bacteriological and chemical analyses, the latter mainly water analyses for municipal departments, and the members of the staff also carried out scientific research.

It was at the suggestion of Sir Henry Roscoe and largely because he had proved himself a successful teacher and lecturer that Harden was appointed a member of the Institute staff. Shortly afterwards, however, the Medical Schools themselves introduced teaching in bacteriology, and the classes at the British Institute were discontinued. In May 1898 the British Institute was transferred to a new building at Chelsea, and later in the year its title was changed to the Jenner Institute of Preventive Medicine.

At the Jenner Institute, Harden was associated with Dr Allan Macfadyen who was in charge of the bacteriological department, and it was at his suggestion that Harden undertook the investigation of the bacterial fermentation of sugars. Originally the object of the work was to find diagnostic tests for the differentiation

of varieties of *Bact. coli commune* and closely related organisms. It was Harden's introduction to the science of biochemistry, and his interest in the nature of bacterial fermentation was maintained during the rest of his life. His first paper on the action of *Bact. coli commune* and allied organisms appeared in the *Transactions of the Jenner Institute* for 1899, and later was extended in the *Transactions of the Chemical Society* [1901, **19**, 610]. He estimated the decomposition products of glucose, compared them with those from mannitol, and established that the two terminal alcohol groups were the source of the acetic acid and alcohol formed in the reaction. *Bacillus lactis aerogenes* was also studied, and the ratio of alcohol to acetic acid produced was shown to serve as a possible basis for the differentiation of these two organisms [*J. Hyg., Camb.*, 1905, **5**, 488]. Two substances, hitherto unknown as products of bacterial action on sugars, 2:3-butylene glycol and acetyl methyl carbinol, were identified as characteristic of *B. lactis aerogenes* [Harden & Walpole, *Proc. roy. Soc.* B, 1906, **77**, 488], and acetyl methyl carbinol was shown to be the substance responsible for the colour test described by Vosges & Proskauer [*Proc. roy. Soc.* B, 1906, **77**, 424]. The bacterial formation of this substance and of the corresponding butylene glycol was followed up in two papers with D. Norris [*Proc. roy. Soc.* B, 1912, **84**, 492; 1912, **85**, 73] and the atomic grouping in proteins which was responsible for the diacetyl test determined [*J. Physiol.* 1911, **42**, 332]. A final paper on this subject appeared dealing with the chemical action on glucose of a variety of *Bact. coli commune* (Escherich) obtained by cultivation in presence of chloroacetate [Penfold & Harden, *Proc. roy. Soc.* B, 1912, **85**, 415].

It was in 1897, the year in which Harden was appointed to his post at the British Institute, that Buchner had published his epoch-making discovery that the living cell was not essential to the process of fermentation and had established that cell-free yeast juice was able to bring about the decomposition of sugar, though the rate of fermentation was greatly diminished. The attention of both bacteriologists and chemists was now focused on yeast, and experiments were started at the Jenner Institute to obtain a yeast antibody. Harden's interest in the subject was aroused, and in 1901 a paper appeared in collaboration with Rowland [*Trans. Chem. Soc.* **79**, 1227] on the autofermentation and liquefaction of yeast. The effect of temperature in hastening liquefaction was studied, and the oxygen absorbed and carbonic acid set free during the process were measured; the conclusion was drawn that the alcoholic fermentation in the liquefied yeast corresponded with the autofermentation of the glycogen it contained, the proportion of alcohol to CO_2 evolved being 1 : 1. This paper is also interesting as being carried out with the assistance of Mr W. J. Young, whose collaboration was to prove so fruitful during the next ten years. With Young, Harden then examined the preparation of yeast glycogen, and made a careful comparison of its properties with those of the glycogens obtained from the oyster and the rabbit [*Trans. Chem. Soc.* 1902, **81**, 1224], and then in 1903 appeared the record of the effect of blood serum in increasing the alcoholic fermentation of yeast juice, the elucidation of which phenomenon was to lead to such fruitful results [*Ber. dtsch. chem. Ges.* 1903, **36**, 715]. The observation that not only did yeast juice exercise no proteolytic effect on rabbit and horse sera but that the latter inhibited the autoproteolysis of the yeast was unexpected, and led to the addition of serum to a mixture of glucose and yeast juice with the striking result that there was an increased evolution of CO_2. The next important step was the discovery that boiled yeast juice though itself incapable of producing fermentation, when added to a fermenting mixture of glucose and yeast juice greatly increased the amount of CO_2 evolved. It had previously been suggested that the

rapid loss of fermentative power characteristic of a yeast juice fermentation was due to the destruction of the alcoholic ferment by a proteolytic enzyme, but Harden and Young attributed the stimulating effect of the boiled yeast juice to an increased activity of the alcoholic ferment and not to the diminution of the proteolytic effect.

In order to follow the progress of the fermentation, Harden and Young measured the evolution of CO_2: they were thus able to follow the rate of the reaction during the whole course of the experiment and to learn much that would have been missed had they dealt only with the end products of the reaction.

In 1903, the Jenner Institute again changed its name and became the Lister Institute, and Dr C. J. Martin was appointed as its Director. In working on the antitoxin of snake venom, Martin had devised a gelatin filter which would work under high pressure and which had been applied by him to the separation of high-molecular colloids from crystalloids. He now suggested its application to the problem of separating the yeast juice into its constituents, and by its use Harden and Young effected the separation into a clear solution and an oily residue. Separately both of these solutions were inactive: together their activity was almost equal to that of the original juice [*Proc. physiol. Soc.* 1904, 32]. A further paper dealt with the differences between the results obtained by Macfadyen, Rowland and Morris and by Buchner in their respective investigations on yeast press juice, and the explanation was found in the differences between the bottom and top yeasts which had been used in the two investigations. In this paper [*Ber. dtsch. chem. Ges.* 1904, **37**, 1052] it was stressed that in both cases a considerable proportion of the sugar was converted into non-reducing substances which were later to be accounted for by the conversion of sugar into polysaccharides [*Biochem. J.* 1913, **7**, 630]. A lecture given to members of the Institute of Brewing in which these researches were described aroused much interest [*J. Inst. Brew.* 1905, **11**, 1].

Between the years 1906 and 1911, Harden and Young contributed six papers to the *Proceedings of the Royal Society* in which were described their classical researches on alcoholic fermentation.

Starting from the fact that the fermentation of glucose by yeast juice was increased by the addition of boiled filtered yeast juice, they showed that the boiled juice could be replaced by the precipitate thrown down by the addition of alcohol to boiled yeast juice, by the liquid formed by autoplasmolysis of yeast, or by the liquid formed by boiling Buchner's acetone-killed yeast with water. By means of the Martin gelatin filter, the juice was separated into two parts, an oily residue, later obtained as a dry powder, and a dialysate containing a thermostable substance. The addition of boiled filtered juice to a mixture of glucose and yeast juice produced a rapid initial evolution of CO_2 gradually diminishing to constant rate; the 'extra' CO_2 was directly proportional to the volume of boiled juice added, and one molecular proportion of CO_2 was given off for each atom of phosphorus added. The fermentation rate then diminished more slowly and continued for a longer period than without the addition of boiled juice. The phosphate entering into the reaction was changed into a form no longer precipitable by the magnesia mixture, yet a solution of soluble phosphate could not replace the boiled juice. Another factor, for which Harden adopted Bertrand's term of 'co-ferment', was also present, both phosphate and co-ferment co-operating with the zymase of the unboiled juice to produce fermentation.

Disappearance either of the ferment or of the co-ferment brought the fermentation to an end, but normally the co-ferment was the first to be used up, dis-

appearing, however, less rapidly in the presence of glucose than in its absence. Addition of phosphate produced the decomposition of an equivalent amount of glucose and a greater total amount of fermentation.

The change of the phosphate to a form no longer precipitable by magnesia mixture was explained when Young [*Proc. Chem. Soc.* 1907, 65] isolated the barium salt of a hexosediphosphoric acid and the demonstration of the slow conversion of the hexosediphosphate into hexose and phosphate by yeast juice was another important step. The equations representing the reactions were given as:

(1) $2C_6H_{12}O_6+2R_2HPO_4=2CO_2+2C_2H_6O+C_6H_{10}O_4(PO_4R_2)_2+2H_2O$,

(2) $C_6H_{10}O_4(PO_4R_2)_2+2H_2O=C_6H_{12}O_6+2R_2HPO_4$.

The co-ferment was regarded as a substance containing a phosphoric group united with a group of unknown composition by means of which the phosphate group was passed on to the sugar molecule and a new phosphate group taken up.

The presence of phosphate inhibited the enzymic decomposition of the hexosediphosphate; an optimum concentration of phosphate produced a maximum initial rate of fermentation and further increase of phosphate diminished the rate of fermentation.

The behaviour of mannose and fructose threw further light on the constitution of the hexosephosphate. Mannose was fermented as rapidly as and fructose more rapidly than glucose. The rates of fermentation of all three sugars were accelerated by phosphate, but that of fructose was double that of glucose or mannose and it was much less rapidly inhibited by the further addition of phosphate. The hexosediphosphates prepared from these three sugars were identical and the quantitative relations were expressed by the same equations. Young suggested that the hexosephosphate was probably derived from the enolic form common to the three sugars, though he did not ignore the possibility that the two molecules of sugar were broken down into smaller groups and hexosephosphate resynthesized from these [*Proc. roy. Soc.* B, 1909, **81**, 528].

After suitable 'training' of the yeast, galactose was also fermented and from this also an active juice was prepared [Harden & Norris, *Proc. roy. Soc.* B, 1910, **82**, 645].

Naturally there followed a period of controversy. Buchner's suggestion that lactic acid was an intermediate in sugar fermentation was rejected by Harden, a view in which Buchner eventually concurred though unfortunately not before it had been widely accepted by biologists. Iwanoff claimed to have isolated a triosephosphate giving an osazone (M.P. 128) similar to one prepared by Lebedeff from the oxidation products of glycerol. According to Iwanoff, the formation of hexosephosphate preceded the alcoholic fermentation, and the amount of fermentation should be proportional to the amount of hexosephosphate decomposed. Harden and Young did not at first accept this view, but the existence of Iwanoff's triosephosphoric acid was confirmed in 1907 by Young who isolated it as a lead salt. No attempt seems, however, to have been made by Harden to introduce the triosephosphate into his equations, and its significance does not appear to have been appreciated. Lebedeff, from the analysis of the osazone, regarded the hexose ester as a monophosphate and challenged the individuality of the hexosediphosphate, suggesting that it was an amorphous mixture of hexosemonophosphate and inorganic phosphate, a suggestion disproved by the discoverers, for Young found that phosphoric acid was liberated when the osazone was formed. Dihydroxyacetone was suggested by Lebedeff as an intermediate stage of glucose fermentation and for a time replaced lactic acid as favourite until it was shown by Harden to be more slowly fermented than sugar;

when added to a fermenting mixture of glucose and yeast juice dihydroxyacetone did not increase fermentation.

Arsenates added to the fermenting mixtures were found greatly to accelerate the production of CO_2 and alcohol, but Harden and Young clearly recognized that arsenate could not be substituted for phosphate and that it played some entirely different role in stimulating fermentation.

For about 25 years the nature of the coenzyme provoked much discussion and research. Harden and Young had correctly described it as a body in which phosphate was combined with some other group which was able to take up and to pass on the phosphate group, but it was only in 1937 that the work of Warburg and of Euler successfully established its structure as that of adenine pyridine diphosphonucleotide.

The partnership of Harden and Young was now broken. Young left for Australia on his appointment as Biochemist to the Australian Institute of Tropical Medicine at Townsville in 1912, and Robert Robison was appointed as his successor. In 1914 the new collaborators announced their discovery of a monohexosephosphate accompanying the hexosediphosphate in yeast; it could be separated by precipitation with basic lead acetate after the diphosphate had been removed by precipitation with normal lead acetate, and was differentiated from the latter by the greater solubility of its barium salt.

When it was discovered that the products of fermentation contained not only a hexosediphosphate but also a monophosphate, in collaboration with F. R. Henley [Lord Henley] Harden again returned to the examination of his equations [*Biochem. J.* 1920, **14**, 642; 1921, **15**, 175] and endeavoured to reconcile them with the presence of both a mono- and a di-phosphate, but they were forced to the conclusion that when a large proportion of monophosphate existed in the solution, the equations could not be applied. The only quantitative relation that could be clearly established was that, during the course of the fermentation, for each molecule of phosphate esterified approximately one molecule of CO_2 was evolved. If dried yeast was used the ratio was slightly greater than unity, with yeast juice or zymin it was somewhat less. The proportions of the mono- and di-phosphates in the solution, however, varied enormously, in the extreme cases consisting of 96% diphosphate or of 86% monophosphate. Obviously the relations of these two intermediate substances were not clearly understood, and it was not until the work of Meyerhof and his colleagues had established that the monophosphate was the first product of phosphorylation and the hexosediphosphate was formed by the phosphorylation of the monophosphate and then converted into two molecules of triosephosphate that any attempt to express the course of the fermentation by equations could have been successful. Harden's equations represented the simultaneous decomposition of two molecules of hexose, only one of which became phosphorylated. He did not for some time accept the view that all hexose molecules became phosphorylated before decomposition took place.

Subsequently it was shown by Macfarlane [*Biochem. J.* 1930, **24**, 1051], working in Harden's laboratory, that an autolysed solution of dried yeast which was incapable of fermenting glucose, even after the addition of the coenzyme, could break down hexosediphosphate producing inorganic phosphate and CO_2. The existence of a definite phosphatase acting on the hexosediphosphate was therefore established. When coenzyme and arsenate were added, the decomposition of the hexosediphosphate was extremely rapid, and CO_2 and inorganic phosphate appeared as final products of the decomposition. It was the breakdown of the hexosediphosphate that was stimulated by the presence of arsenate.

Of the great fall in fermentative activity which occurred when fresh yeast was converted into yeast juice, 80% was found to occur during the process of grinding, and it was during the same period that the yeast acquired the power of responding to phosphates. It was therefore the phosphatase which was affected [Harden & Macfarlane, *Biochem. J.* 1930, **24**, 243].

The nature of the enzymes in yeast and their method of action was investigated by Harden and his colleagues in a long series of researches which were more especially concerned with the enzymes existing in the dried yeast preparations.

He confirmed Neuberg's discovery [Neuberg & Hildesheimer, *Biochem. Z.* **31**, 170] of the existence of the enzyme carboxylase in dried yeast which split pyruvic acid into CO_2 and acetaldehyde and showed that as this reaction took place after the coenzyme had been completely washed away, it was independent of the presence of the coenzyme [*Biochem. J.* 1913, **7**, 214]. The reducing power of zymin or dried yeast, on the other hand, was removed by washing but could be restored by the addition of the boiled washings. Certain aldehydes also restored the reducing power and therefore had the property of acting as oxygen acceptors. Similar experiments carried out with washed rabbit muscle showed that here the addition of either lactic acid or acetaldehyde restored the reducing power [Harden & Norris, *Biochem. J.* 1914, **8**, 100; 1915, **9**, 330]. Evidence was obtained of the existence of a peroxidase in fresh yeast, the activity of which was lost when the yeast was dried for 17 hr. at 37°, but was restored by washing. Washing the dried yeast removed the activity of the invertase and maltase, whereas that of the catalase was not affected [Harden & Zilva, *Biochem. J.* 1914, **8**, 222]. Harden also showed that the presence of inorganic salts exercised a marked influence on the autofermentation of yeast [Harden & Paine, *Proc. roy. Soc.* B, 1911, **84**, 448], since all dissolved substances which plasmolysed the yeast cell caused also a large increase in the rate of autofermentation. The study of the addition of substances capable of acting as oxygen acceptors led to a surprising result. Washed zymin in the presence of a suitable concentration of sodium phosphate was activated by sodium pyruvate but not by the acetaldehyde into which the pyruvate was decarboxylated by yeast; if, however, the sodium phosphate were replaced by the potassium salt, the acetaldehyde also acted as an activator. Obviously the sodium and potassium ions were not interchangeable, and the potassium ion played some specific role [*Biochem. J.* 1917, **11**, 64]. On the other hand, when the fermentation of fructose or glucose by dried preparations of yeast was examined, it was found to be equally depressed by the addition of sodium or potassium chloride, the effect of the sulphates being somewhat greater. This effect was traced to the action of the salts on the hexosephosphate reaction. Potassium phosphate also produced a depressing effect, but here the depressing effect could be counteracted by the addition of acetaldehyde to the solution [Harden & Henley, *Biochem. J.* 1921, **15**, 312].

When dried yeast preparations acted in the presence of a large volume of sugar solution there was a considerable induction period before rapid fermentation set in, this period being reduced by the addition of various salts, both organic and inorganic. Sodium arsenate alone prolonged the time of the induction period without any toxic effect on the subsequent fermentation [Harden, *Biochem. J.* 1925, **19**, 477; Harden & Macfarlane, 1928, **22**, 786]. It was not possible to explain satisfactorily the effect of the ions, but the complexity of the process became ever more apparent.

War broke out in 1914, and from 1915 during the absence of the Director on military duties, Harden acted as Deputy-Director of the Lister Institute. About

three years previously, at the suggestion of Dr C. J. Martin, work on the nature of the substance responsible for curing the polyneuritis of pigeons had been carried out at the Lister Institute by Funk and Cooper, and in 1917, when the subject had become one of great practical importance, Harden in collaboration with Zilva carried out a series of researches on the antineuritic and antiscorbutic vitamins. They established that neither α-hydroxypyridine nor adenine was the active agent in curing beriberi as had been previously suggested [*Biochem. J.* 1917, **11**, 172]; they differentiated clearly between these two water-soluble factors showing that the antineuritic but not the antiscorbutic substance was adsorbed by fuller's earth [*Biochem. J.* 1918, **12**, 93]. A considerable concentration of the antiscorbutic factor was achieved by removing the organic acids of lemon juice, leaving a decitrated residue of much increased potency [*Biochem. J.* 1918, **12**, 250], and in collaboration with Dr Still the clinical effect of this product was studied. Harden and Zilva were the first to recognize clearly that scurvy was a disease brought about by the dietetic deficiency of a specific substance and not, as had been suggested by MacCollum and Pitz, by a generally unwholesome diet. The susceptibility of this vitamin to alkalinity was noted [*Lancet*, 7 Sept. 1918]: the antineuritic and antiscorbutic principles were shown to be lacking in ale, stout and fine beer. The antiscorbutic requirements of the monkey were determined [*J. Path. Bact.* 1919, **22**, 246; *Biochem. J.* 1920, **14**, 171; *Lancet*, 1919, **2**, 7, 780], and the antineuritic substance shown to be necessary for the growth of frogs [*Biochem. J.* 1920, **14**, 263]. With Bacot, *Drosophila* was shown to need B but not the C vitamin [*Biochem. J.* 1922, **16**, 48], and evidence was given establishing the biological synthesis of vitamin B by yeast [*Biochem. J.* 1921, **15**, 438]. With Robison, the practical effect on their vitamin C content of heating and storing fruit juices was investigated and results of practical importance obtained which were published in the *Journal of the R.A.M.C.* [*Biochem. J.* 1920, **14**, 171]. A Friday evening lecture on this subject was given at the Royal Institution on 28 April 1922.

During this period Harden did valuable work as a member of the Accessory Food Factors Commmittee set up by the Medical Research Council and was the editor of their Report on this subject.

The chemist who brings about chemical transformations in the laboratory relies largely on splitting off and recombining the elements of water. Here he can work at high temperatures and with powerful reagents. In the laboratory of the living organism the reactions must be brought about at almost constant temperature and with no violent changes of reaction. It is the great achievement of Harden and his colleagues to have discovered that the process of phosphorylation and dephosphorylation forms the basis of the mechanism by which carbohydrate is broken down in the living organism. They elucidated the manner in which this process was brought about, since they discovered that a definite substance, 'the coenzyme', existed and suggested that its function was to take up and pass on the phosphate radical. Their evidence of phosphorylation was indisputable since they isolated two intermediate compounds in which phosphate was bound to the hexose molecule, the 1:6-hexosediphosphate and the 6-hexosemonophosphate. Evidence of the process of dephosphorylation was given when they described the effects of a specific enzyme, a hexosediphosphatase, breaking down the hexosediphosphate with liberation of free phosphoric acid. The foundations of carbohydrate biochemistry were indeed well and truly laid.

Harden's power of accurate observation and of clear thinking enabled him to find his way in this new and complicated field so that although there remained many steps to fill in, there was little to withdraw. He realized early the com-

plexity of the process himself, describing the yeast cell as provided with a regular army of enzymes and coenzymes.

This work of Harden has already had two remarkable developments. It was applied to unravel the chemistry of the breakdown of carbohydrate in muscle by Embden in 1913 and later by Meyerhof and his colleagues, and as the result the steps by which glycogen is converted into lactic acid in the animal organism have been elucidated. From his work with Harden on yeast monophosphate Robison was led to his studies on the nature of ossification, where again the process of phosphorylation and dephosphorylation was found to be involved.

Recognition of the importance of his researches was not long delayed. Harden was elected to the Fellowship of the Royal Society in 1909. He was made Head of the newly formed Department of Biochemistry of the Lister Institute in 1907 and Professor of Biochemistry in the University of London in 1912. In 1929 he shared with Euler the Nobel prize for biochemistry. He was awarded the Davy Medal of the Royal Society in 1935 and in 1936 received the honour of knighthood. The Institute of Brewing made him an Honorary Member, the Universities of Manchester, Liverpool and Athens conferred Honorary Degrees upon him, and the Kaiserlich Leopold Deutsche Akademie der Naturforsche, Halle, elected him to its membership. The award of the Nobel prize gave him much pleasure and he enjoyed greatly the Stockholm visit. It was fitting that at the same ceremony Sir Frederick Gowland Hopkins shared with Eijkman the Nobel prize for Medicine, and that the two men who had done so much to build up the science of biochemistry should thus have been honoured together.

At the Lister Institute there was little opportunity for Harden to exercise his undoubted gift for teaching, but for many years one evening a week was devoted to a class in microbiology, at the Sir John Cass Institute, in which he took the greatest interest. Literary work took up a good deal of his time. Beside undertaking the very onerous work as editor of the *Biochemical Journal*, he wrote the article on 'Fermentation' for Thorpe's *Dictionary of Chemistry*, the section on 'Yeast enzymes' for Oppenheimer-Pincussen's *Die Fermente und ihre Wirkungen*, and the section on alcoholic fermentation, 'The early stages of fermentation in the yeast cell', for *Die Ergebnisse der Enzymforschung* [Leipzig, 1932]; he was the author of *Alcoholic Fermentation* in the Longmans series of Biochemical Monographs, of which four editions have already appeared, and of the article on 'Bacterial Metabolism in the System of Bacteriology' published by the Medical Research Council [1930].

He was an excellent Committee member, and as editor was a member of the Committee of the Biochemical Society for more than 25 years. He also served on the Council of the Chemical Society, of which he became a Vice-President, and was for some years a member of the Research Fund Committee of the Institute of Brewing, and from 1930 to 1938 Chairman of that Institute's Advisory Committee on Yeast.

Biochemists owe much to Harden not only for the part he took in opening up a large field of new and important work but also for his efforts in securing the recognition of the science of biochemistry in this country. He was one of the founders of the Biochemical Society in 1911, and when in 1912 that Society acquired from Prof. Benjamin Moore the proprietorship of the *Biochemical Journal*, Harden accepted with Bayliss the office of editor. The latter, however, consented to act as joint editor only on condition that he should not be asked to do proof reading, and until the death of Bayliss, twelve years later, the whole brunt of this most arduous work fell on Harden. In 1924 H. W. Dudley became joint editor to be succeeded in 1930 by C. R. Harington, and in this period the

work was more equally divided. In 1938, after Harden had acted as editor for 25 years, the Society expressed their appreciation of the debt they owed him by presenting him with a silver salver on which were engraved the signatures of all the Committee members of the Biochemical Society who had served with him. The success of the *Journal* and the appreciation of his colleagues were for many years the only reward of his unselfish work. It was indeed a notable contribution to the development of the science of biochemistry in this country. He attended meetings of the International Congress of Chemistry, playing an important part on the Committee for the Reform of Biochemical Nomenclature.

Harden had many interests. He kept in touch with his old Manchester friends and students, amongst them Dr H. G. Colman, Prof. S. P. Bedson, Sir Philip Hartog and Sir John Russell. He was a delightful friend, always cheerful and with a dry sense of humour. He read widely and was well versed in the works of the Victorian novelists, liking especially Dickens and Trollope. He enjoyed visits to the theatre and opera, rather as pleasant social functions than because of any deep interest in drama or music. In his later years he was fond of travel and made tours to Greece and Spitzbergen and went with the British Association to South Africa in 1935, keeping on each occasion a notebook in which his doings were recorded and objects of interest were tersely described.

He was an excellent skater, and when a hard frost set in was not expected in the laboratory. In his earlier days at the Lister Institute, with several of his colleagues he joined the Wimbledon Park Golf Club, and many friendly matches were arranged in which Harden's somewhat unorthodox stance used to come in for criticism. During the war of 1914–18 he joined the Volunteer Reserve, and his leisure was spent in digging trenches and in other military exercises. After the war he never returned to golf, but took exercise in gardening. Outside his work his greatest interest was in his garden, and in the summer he generally came to the Institute with some choice bloom in his buttonhole. He was a Fellow of the Horticultural Society, a regular attendant at their flower shows, and his knowledge of flowers was extensive. With his colleagues, Ledingham, Arkwright and Robison, Harden took the keenest interest in the *Observer* acrostics. Every Monday morning, notes were compared, and if a solution was incomplete or dubious, plans were laid for more serious inquiry before the last post on Wednesday.

Harden was a man of methodical habit and of even temperament, knowing neither the heights nor depths of emotion. His philosophical outlook, sound judgement and critical instinct were much valued by his colleagues, and when his advice and help were asked they were readily given, but he was a man whom it was difficult to know intimately. His outlook on life was detached, and he once said of himself at a time of personal sorrow that he had never known what it was to be elated or depressed. His friends regarded him as a man to be thoroughly liked, trusted and respected. His students found him always pleasant to work with and sometimes unexpectedly encouraging when difficulties arose. In some respects he resembled the typical idea of the Englishman, showing neither enthusiasm nor emotion and performing as a matter of course what seemed to him to be his duty, without thought of reward, even when this involved much personal labour freely given. He preferred to be rather an onlooker than a participant in a struggle, and this detachment probably prevented him from entering into closer relations with his students and fellow-workers.

In 1900, he married Georgina Sydney (daughter of Mr C. W. Bridge of Christchurch, New Zealand) who died in 1928. They lived for some time at Richmond and later moved to Bourne End, and at both places his friends and students were entertained to pleasant river excursions in the summer.

In 1930 he retired from his Professorship at the Lister Institute but continued to come every day to his laboratory carrying on experimental work and correcting proofs until a year or two before his death. Towards the end of his life he suffered from a progressive disease from which release came before it had become too irksome and while he still enjoyed his garden, his books and his interest in science.

IDA SMEDLEY-MACLEAN

ROBERT ROBISON

(1883–1941)

It is sad to record the death of Robert Robison who died suddenly in his 58th year. His friends in the Biochemical Society and his colleagues at the Lister Institute are left to mourn the loss of a man of rare charm and fine character.

Robison was born at Newark-on-Trent in 1883 and obtained his early education at the Magnus Grammar School in his native town. He entered University College, Nottingham, in 1900 and attended the usual course of study for an honours degree in chemistry. A breakdown in health, however, caused him to postpone his final examination, and he actually commenced research some time before he finally graduated B.Sc. with honours in chemistry. For two years he investigated the organic compounds of silicon under the direction of Prof. F. S. Kipping, and as a result of this work, which was mainly on benzyl and benzylmethyl derivatives of silicon [Robison & Kipping, *J. Chem. Soc.* 1908, **93**, 439], he was awarded, in 1907, an '1851 Exhibition' Scholarship. The next two years were spent in the laboratory of Prof. Hantzsch at Leipzig, where Robison carried out investigations bearing on the relationship between the colour and constitution of organic compounds. The results of these researches were embodied in two papers [Hantzsch & Robison, *Ber. dtsch. chem. Ges.* 1910, **43**, 45, 92], and the degree of Ph.D. [*summa cum laude*] was awarded to him for his thesis entitled 'Über die polychromen Salze aus Dimethyl und Diphenyl-Violursäure'.

A fellow-student in Leipzig at that time tells us that Robison appeared to enjoy every minute of this stay there. He was fond of music, visited the *Gewandhaus* frequently, and made a special point of being present in the Opera House when his favourite works were given. Being a keen walker he made many excursions during the vacations to other parts of Germany, Austria, Italy and Switzerland.

Robison returned to England in the summer of 1909, and for some months worked in the laboratory of the Nottingham City Analyst, but towards the end of the year he was appointed lecturer and demonstrator in chemistry at University College, Galway. Here much of his time was taken up with plans and arrangements for new laboratories, and although he started work on possible methods for the synthesis of acridines, in collaboration with the late Prof. Senier, he did not complete the investigation, as in October 1910 he was appointed to the post of lecturer and demonstrator in chemistry at University College, Nottingham. The work on organic compounds of silicon was resumed in collaboration with Prof. Kipping, and during the next three years four publications on this subject appeared [*J. Chem. Soc.* 1912, **101**, 2142, 2156; 1914, **105**, 40, 984].

Robison was appointed an assistant in the Biochemical Department of the Lister Institute of Preventive Medicine in March 1913, and here he made his first contact with biochemical problems. He remained a member of the Institute's

Staff from this time until his death. Robison commenced work with characteristic thoroughness and enthusiasm and, in collaboration with his new chief, Prof. Harden, was soon able to announce, in a preliminary note [Harden & Robison, *Proc. Chem. Soc.* 1914, **30**, 16] the discovery of a new hexosephosphoric ester, which, although not at that time obtained in a pure condition, was identified as a hexosemonophosphate. This ester, or more correctly, mixture of esters, was later known as the 'Robison Ester', and was destined to play an all-important part in the post-war development of biochemistry.

The War caused a complete break in Robison's work which lasted for the four years 1914–18. In December 1914 he enlisted in the Sanitary Section of the Royal Army Medical Corps, and after a short period of training at home was placed in charge of a sanitary unit and left for Egypt with the 29th Division early in 1915. Soon after his arrival at Alexandria he became ill with dysentery and, although still far from well, he proceeded a few weeks later to Mudros where he was stationed during the whole of the campaign in Gallipoli. During this period his general health suffered considerably, but on his return to Egypt he spent some months doing general hygiene work at El Kantara before giving up and going into hospital at Alexandria. He was finally invalided home early in 1917. With his return to health later in the same year Robison was posted with the rank of captain to a hygiene unit laboratory attached to the Italian Expeditionary Force; he remained in Italy until some months after the armistice in 1918.

After his return to the Lister Institute, Robison at first collaborated with Harden in an investigation of the antiscorbutic properties of concentrated fruit juices [*J. R. Army Med. Cps*, 1919, **48**, 53; *Biochem. J.* 1920, **14**, 171; 1921, **15**, 521], and the nature of the sulphuric acid tests for liver oils [*Biochem. J.* 1923, **17**, 115]. Subsequently he worked with C. J. Martin on the biological value of proteins in milk and wheat [Martin & Robison, *Biochem. J.* 1922, **16**, 407] and alone on that of gelatin [*Biochem. J.* 1922, **16**, 111], and he availed himself of this opportunity to study the distribution of the nitrogenous constituents of the urine on a diet containing a minimal amount of nitrogen [*Biochem. J.* 1922, **16**, 131]. The experiments, made on themselves, were rather severe on the digestive machinery of the workers, and Robison had to abandon them on account of a recurrence of his old intestinal trouble and had to go to hospital for repair. On restoration to reasonable health he reverted to an investigation on the hexosemonophosphoric ester he had previously described with Harden in 1914. The isolation and purification of the ester proved to be a difficult problem, but Robison finally published an account of this important work [*Biochem. J.* 1922, **16**, 809], and immediately proceeded to examine in detail the chemical nature of the products of yeast preparations on simple sugars in the presence of inorganic phosphate; the results of this work were published some years later [Robison & Morgan, *Biochem. J.* 1930, **24**, 1905]. In a series of papers in collaboration with numerous co-workers, he was able during the following years to record the isolation and identification of trehalosemonophosphoric ester [Robison & Morgan, *Biochem. J.* 1928, **22**, 1277], glucose-6-phosphate [Robison & King, *Biochem. J.* 1931, **25**, 323], mannose-6-monophosphate [Robison, *Biochem. J.* 1932, **26**, 219; Robison & Jephcott, 1934, **28**, 1844], fructose-6-phosphate [Macleod & Robison, *Biochem. J.* 1933, **27**, 286], fructose-1-phosphate [Tankó & Robison, *Biochem. J.* 1935, **29**, 961]; and finally an unidentified phosphoric ester [Robison, Macfarlane & Tazelaar, *Nature, Lond.*, 1938, **142**, 114], the sugar component of which resembles in many ways manno-ketoheptose, a 7-carbon atom sugar that was isolated from the avocado pear by La Forge. A close examination

of these papers shows the tenacity and resolution with which Robison carried out his investigations, and up to the time of his death he was still actively engaged on the isolation of certain unidentified sugar phosphoric esters.

Early in 1923 Robison discovered the enzyme, phosphatase, in the aqueous extracts of bones of young rapidly growing animals and worked out a method for obtaining very active phosphatase preparations which could hydrolyse many different monosubstituted phosphoric esters but which had only slight action on disubstituted esters such as diethylphosphate, and little or no hydrolytic action on lecithin. Robison used the bone phosphatase as a biochemical reagent in several investigations of which those dealing with the removal of the phosphoric acid groups from the methylated phosphoric esters of hexose di- and monophosphates [Morgan & Robison, *Biochem. J.* 1928, **22**, 1270; King, McLaughlin & Morgan, *Biochem. J.* 1931, **25**, 310], the isolation of crystalline trehalose from trehalosemonophosphate [Robison & Morgan, *Biochem. J.* 1928, **22**, 1277] and the preparation of gluconic acid from the phosphogluconate obtained by the bromine oxidation of glucosemonophosphate [Robison & King, *Biochem. J.* 1931, **25**, 323] are interesting examples and are sufficient to show the importance of this method of attack on the chemical constitution of the sugar phosphoric esters.

In some of his earlier experiments Robison observed that during the hydrolysis of fructosediphosphate (1:6-diphosphofructofuranose) by preparations of the bone phosphatase, a part of the sugars liberated, following the removal of the phosphoric acid groups, suffered an intramolecular change and gave rise to a mixture of aldose and ketose sugars [Martland & Robison, *Biochem. J.* 1929, **23**, 237], and similar results were also obtained with the glucosemonophosphoric ester [Robison & King, *Biochem. J.* 1931, **25**, 323]. A more detailed study of these changes was undertaken later [Macleod & Robison, *Biochem. J.* 1933, **27**, 286], and the investigation soon revealed that the proportion of aldose formed during the hydrolysis varied with the phosphatase preparation employed. In a final communication in collaboration with Tankó [*Biochem. J.* 1935, **29**, 961] he later announced the discovery of a phosphohexosekinase. The enzyme was isolated from crude preparations of bone phosphatase and from bone marrow extracts and appeared to be similar to the enzyme discovered by Lohmann in muscle, kidney, liver and dried yeast which converts the Neuberg ester into an equilibrium mixture of aldose and ketose esters. Robison finally showed that the phosphohexosekinase converts fructose-6-phosphate and glucose-6-phosphate into equilibrium mixtures of aldo- and keto-esters, and demonstrated that the enzyme had no action on fructose-1-phosphate or on 1:6-fructosediphosphate unless phosphatase was present and first removed the phosphate group from position 1.

Although Robison never engaged directly in the problems of muscle glycolysis he was much interested in the advances made in this field, more particularly when the proposed scheme of glycogen breakdown or synthesis involved the various hexosephosphoric acids and enzyme systems he himself employed in the course of his work. As one would anticipate from a knowledge of his generous character, the brilliant achievements of others, especially those investigators whose work dealt with the breakdown of glycogen through the intermediate formation of glucopyranose-1-phosphate and its subsequent transformation into glucopyranose-6-phosphate, and the demonstration that enzymically active preparations of animal and vegetable tissue could bring about a synthesis of glycogen from pyranose-1-phosphate, gave him great satisfaction and made him feel that his own findings were becoming of increasing value to others in the general problem of polysaccharide breakdown and synthesis.

The most outstanding contribution made by Robison in this field was the isolation of 'hexosemonophosphoric ester', or as it is now generally called, the Robison ester. Otto Warburg, in a recent article on the 'Chemische Konstitution von Fermenten', leaves no doubt as to the importance of this part of Robison's work as the following passage shows: 'Robisonester ist in den Testen, durch die das gelbe Ferment, die beiden Pyridin-Nucleotide und das Zwischenferment isoliert worden sind, das Substrat gewesen. Wahrscheinlich wäre heute keine dieser Substanzen isoliert, hätte der Robisonester nicht zur Verfügung gestanden. Dass wir nachträglich auch Alkohol als Test-Substrat benutzt haben, ändert nichts an dem historischen Gang der Dinge.'

During these busy years of Robison's life he gave several evening courses in biochemistry at Chelsea Polytechnic. The lectures and practical classes were carried through with his characteristic attention to detail, and the writer, who acted as demonstrator at that time, remembers vividly the care that was taken during the afternoon preceding the evening class to make certain that the experiment to be carried out by the students could be completed in the short time at their disposal. With experiments involving the use of enzymes—the proteinase of fresh pine-apple juice or the phosphatase of bone extract—no effort was spared to ensure that the enzyme hydrolysis which was planned to commence at a few minutes past 8 o'clock would be complete by 9.30 p.m. The students would be astonished with the rate and extent of hydrolysis accomplished in so short a time, little realizing that their successful experiment was largely due to the very careful and thorough preparation made by Robison before the class commenced!

Perhaps Robison's most famous work lay in his researches on the calcification of cartilage in bone formation, the significance of which was capable of appreciation by a larger circle. During a chance talk with C. J. Martin, then Director of the Lister Institute, on the pathology of rickets—a subject that was being investigated with vigour in the Institute at that time—Robison learned that it was not known why cartilage that showed the histological changes preceding ossification had, under normal circumstances, bone salt subsequently deposited in it. Robison considered the matter for a few days and then suggested that there must be some mechanism whereby the solubility product of calcium phosphate was locally increased, thus causing a deposition of the salt. To account for the increase he suggested a liberation of phosphate ions from a phosphoric ester contained in the blood and pointed out that if these ideas were correct there must be a local supply of a phosphatase in the ossifying cartilage. Robison had observed, while studying the action of the enzyme, emulsin, on the readily soluble barium salt of hexosemonophosphoric ester, that the inorganic phosphate liberated by the enzyme was immediately precipitated as insoluble barium phosphate, and he was thus in a position at this time to appreciate at once the wider significance of the observation. The next step was to obtain direct experimental evidence for the presence of phosphatase in cartilage that was about to calcify, and this he did by placing the bones of young rats in solutions of calcium hexosemonophosphate. In the course of a few hours a deposit of calcium phosphate was formed, and thus his original suggestion seemed to be correct. Further experiments carried out with the proper controls soon confirmed the fact that ossifying cartilage does indeed contain a very active phosphatase. The results of this work [Robison, *Biochem. J.* 1923, **17**, 286] opened up a new field of investigation which, from a biochemical standpoint was, at that time, almost untouched. The discovery of an enzyme in the ossifying cartilage of young rats and rabbits which rapidly hydrolysed hexosemonophosphate yielding inorganic

phosphate, led to many investigations on the isolation, the mode of action and the distribution of this phosphoric esterase in different tissues [Robison & Soames, *Biochem. J.* 1924, **18**, 740; Kay & Robison, *Biochem. J.* 1924, **18**, 755; Martland & Robison, *Biochem. J.* 1927, **21**, 665; 1929, **23**, 237; Macfarlane, Patterson & Robison, *Biochem. J.* 1934, **28**, 720]. An examination of cartilage and of bones in early stages of development showed that the enzyme which hydrolyses monophosphoric esters is not present in any cartilage prior to the appearance of a centre of ossification, but is found wherever active ossification is taking place. Robison concluded that the production of the enzyme, phosphatase, is a part of those cellular activities which result in the formation of bone. Robison & Soames [*Biochem. J.* 1930, **24**, 1922] developed a special technique whereby it was possible to follow the deposition of calcium phosphate in bone slices taken from rachitic rats, when these were immersed in calcium hexosemonophosphate or glycerophosphate at *p*H 8·4–9·4, and a careful examination of the bone tissue during this artificial ossification process indicated that the phosphatase is secreted in the region of the osteoblasts and hypertrophic cartilage cells.

Robison's theory of bone calcification demanded that there should be a local increase in the concentration of phosphate ions which would cause the solubility product of relatively insoluble calcium phosphate to be exceeded and a deposition in or around the cells containing the enzyme to take place. Robison realized that the occurrence in bone and ossifying cartilage of an enzyme which hydrolysed hexosemonophosphoric esters is in itself not sufficient evidence that esters of this type are concerned in the process of ossification. He realized that such esters must be shown to be present in the blood and to be available wherever calcium phosphate is being deposited. Experiments designed to show that this condition was probably fulfilled were made by Kay & Robison [*Biochem. J.* 1924, **18**, 757] who showed that at least two forms of acid-soluble phosphoric esters were present in the blood and that one of these esters was rapidly hydrolysed by the enzyme of ossifying cartilage. The hydrolysable ester was found to be present almost entirely in the red corpuscles of the blood and although their experiments indicated that these cells might function as a reservoir of the phosphoric ester, which was subsequently found in the blood plasma in small amounts [Martland & Robison, *Biochem. J.* 1926, **20**, 847], it was never demonstrated that the ester could diffuse out of the erythrocytes and thus be available as a substrate for the bone phosphatase. Quite early in this work Goodwin & Robison [*Biochem. J.* 1924, **18**, 1161] succeeded in isolating from the total organic phosphate of the blood a fraction that was very similar in properties to the yeast hexosemonophosphate. In later years Robison collected a large quantity of this organic phosphate of blood with the intention of returning to the attack on the exact nature of the ester but this was never carried out.

As the work on different aspects of the problem of ossification developed Robison became convinced that the simple scheme originally put forward provided an inadequate picture of the normal process of ossification. Why did such tissues as intestinal mucosa, mammary gland and kidney, tissues rich in phosphatase, fail to calcify? Why does rachitic cartilage which possesses an abundance of phosphatase not ossify? It seemed that hypertrophic cartilage and osteoid are different from other tissues in that they possess some special property that enables them to initiate the local deposition of bone salt. He gave much thought to these questions and as a result of a series of carefully planned experiments [Robison, Macleod & Rosenheim, *Biochem. J.* 1930, **24**, 1927; Robison & Rosenheim, *Biochem. J.* 1934, **28**, 684] demonstrated the presence of a 'second'

mechanism. These experiments showed that the mechanism which effects calcification in inorganic solutions (containing no P-ester) is seriously damaged by chloroform or alcohol, by desiccation, by cyanide, iodoacetate or fluoride and other agencies, all of which leave the phosphatase mechanism unimpaired. The results led Robison and Rosenheim to conclude that the full calcifying mechanism may consist of a complex enzyme system of which phosphatase is one component. According to this view the bone may itself be able to synthesize phosphoric esters from inorganic phosphate, these esters being subsequently hydrolysed at the site of calcification. From this time on, the dual nature of the process of calcification has been generally recognized.

In collaboration with Miss Fell, Robison investigated the growth, development and phosphatase activity of the embryonic avian femora and limb buds cultivated *in vitro* and showed that during growth *in vitro* the isolated femora synthesized phosphatase, which increased with histological differentiation [Fell & Robison, *Biochem. J.* 1929, **23**, 767]. The results of these experiments on the development *in vitro* of skeletal tissue confirmed the view that phosphatase is synthesized by cartilage, by the hypertrophic cells found in the preliminary stage of ossification. In a further study [Fell & Robison, *Biochem. J.* 1930, **24**, 1905] the phosphatase activity of the mandibular skeletal tissue of the embryonic fowl was investigated and it was shown that in the normal embryonic palatquadrates, as in femur, the progressive development of hypertrophic cartilage cells and of osteoid tissues was accompanied by rapidly increasing phosphatase activity, while the non-ossifying cartilage from Meckel's rod remained inactive in early post-embryonic life.

Finally, in a series of brilliantly designed and carefully controlled experiments, Fell & Robison [*Biochem. J.* 1934, **28**, 2243] showed that the developing osteoid tissue and hypertrophic cartilage cannot at once acquire the complete calcifying mechanism but that this mechanism is gradually developed during the course of tissue differentiation. Other experiments with normal embryonic tissues [Niven & Robison, *Biochem. J.* 1934, **28**, 2237] showed that the hypertrophic cartilage in the rudimentary long bones becomes calcified shortly after it first appears and if removed from the embryo before deposition has taken place the hypertrophic cartilage can be completely calcified *in vitro*.

Robison's last papers on this subject were concerned with the deposition of strontium salts in hypertrophic cartilage *in vitro* [Robison, Law & Rosenheim, *Biochem. J.* 1936, **30**, 66] and on the influence of changes induced by cholesterol upon the calcification *in vitro* of rabbit aorta [Law & Robison, *Biochem. J.* 1936, **30**, 69].

It will be seen that for several years before his untimely death Robison published nothing on the subject of calcification, but during this period he thought much on the problem and new ideas continually emerged. The simple conception of calcification originally put forward by him slowly became more complex in order to include the later experimental facts and it was well known to his colleagues that Robison was dissatisfied with the state of knowledge on this important subject and that he intended, as soon as conditions once again became normal, to resume his attack on the problem.

Robison was elected a Fellow of the Royal Society in 1930 and was appointed, some months later, head of the Biochemical Department of the Lister Institute and Professor of Biochemistry in the University of London in succession to his former chief, Sir Arthur Harden. In 1931 Robison was invited to deliver the Herter Lectures on Pathological Chemistry in the New York University and Bellevue Medical College, and he subsequently published the substance of these

lectures in book form under the title *The Significance of Phosphoric Esters in Metabolism*. The book gives in clear and simple language an excellent account of his work up to 1932. In 1933 Robison was awarded the Baly Medal of the Royal College of Physicians, an honour he greatly valued.

Robison gave good service to the Biochemical Society as Honorary Secretary from 1926 to 1930, Member of the Committee from 1931 to 1934 and Chairman during the year 1935. His friendly and sympathetic personality will be sorely missed at our Meetings.

Those who came in contact with Robison in his daily life must have realized that few men could have shown such whole-hearted devotion and enthusiasm for their subject. The solution of a problem was its own sufficient reward. It would be difficult to imagine a more stimulating and conscientious director of a research department. Indeed, his conscience sometimes diminished his effectiveness by exhausting his supply of energy. Robison was held in great affection by many and more especially by those younger workers, who, while working at the Lister Institute, were privileged to gain his confidence. Robison had many interests beyond the realms of science and showed a strong inclination towards the arts. His interest in music, more especially in German opera and 'Lieder', was sincere, while his reading of German literature, cultivated with care during his stay in Leipzig, was never allowed to lapse. He was an active gardener and found especial pleasure in this pursuit during the present difficult years.

Robison passed away in his sleep on 18 June 1941. With all the suffering he had seen around him in London during the last year of his life he had frequently expressed a wish that the end might come in this way. The man has gone from us, but the charm of his personality and the high quality of his scientific work will always be remembered by his colleagues who greatly deplore his passing and extend their sympathy to his widow and daughter who survive him.

W. T. J. M.

123. STUDIES IN THE CHEMISTRY OF THE INSECT CUTICLE

I. SOME GENERAL OBSERVATIONS ON CERTAIN ARTHROPOD CUTICLES WITH SPECIAL REFERENCE TO THE CHARACTERIZATION OF THE PROTEINS

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Previous workers have shown that, besides the nitrogenous polysaccharide chitin, the cuticle of insects contains high proportions of other substances, including protein. In his classical survey of the insect cuticle Odier [1823] recognized five general categories of substances in the elytra of cockchafers and other insects; an inert substance, which he proposed to call chitin, pigments, fatty material, albuminous substance and mineral components. Of these the chitin proper formed the skeletal structure and was, in the cases investigated, impregnated with large quantities of brown-pigmented protein removable by heating with potash. This was overlain by a thin layer of fat-soluble material often containing brightly coloured pigments.

Among the large number of chitin-containing organs of both plants and animals two main characters are apparent. (1) As far as they have been studied all specimens of chitin have been shown to be almost identical, both chemically and crystallographically. (2) The chitin always occurs associated with protein. The chitin itself has been studied very closely and is known to be composed of chains of condensed *N*-acetyl glucosamine units and is identical with cellulose except in that the secondary —OH at C_2 is substituted by an acetamide group. Krawkow [1892] showed that chitin is present in all groups of Arthropods and in certain Annelids and the Cephalopod Molluscs. Further, he showed that wherever chitin structures occur protein is also present. Iwanoff [1923] obtained chitin from fungi and showed that protein was associated with it, probably through an organic phosphate linkage. Gonell [1926] suggested from a review of the X-ray diffraction properties of a large number of chitin preparations from plant and animal sources that their micro-crystalline structures are, with rare exceptions, identical. Campbell [1929] made a quantitative investigation of the chitin content of the insect integument, using a method based on the van Wisselingh reaction; in the cockroach he found large variations (e.g. exocuticle 23% chitin, endocuticle 57% chitin).

In most of the above-mentioned studies attention was focussed on the chitin proper. Wigglesworth [1933] obtained qualitative evidence for the presence of protein in the cuticle of *Rhodnius* (Hemiptera) at several different stages of the life history. Pryor [1940] obtained similar data for other insects. Fraenkel & Rudall [1940] obtained some general quantitative data on the composition of the cuticle of the blowflies *Sarcophaga falculata* and *Calliphora erythrocephala* and using X-ray analysis threw light on the distribution and function of protein

in the general structure. They showed that 60% of the dry weight of the larval cuticle of *Sarcophaga* and 47% of the puparium is chitin. In both cases the non-chitin material has the typically high N content of protein.

Apart from its academic interest the insect cuticle has been studied in the present work with a view to contributing to the elucidation of certain physiological problems. These are concerned with its permeability as a membrane and in particular with the penetration of a large group of insecticidal agents which are known to act through the cuticle.

It has now been possible to characterize the proteins of the larval cuticle of *Sarcophaga*; the first is water- and borate-soluble, the second is extracted from the residue by 5% NaOH and contains carbohydrate. Similar fractions have been prepared from the hawk moth (*Sphinx ligustri*) and a water-soluble protein from the cuticle of the lobster (*Homarus*). Certain specific differences in the compositions of the proteins from the three sources have been noted. It appears that the hard melanized protein of the puparium of *Sarcophaga* is similar to the soluble protein of the larval cuticle, and leads to agreement with the view that the correlated hardening and darkening of the puparium is due to the tanning of the protein-chitin complex by diphenolic and quinonoid substances derived from tyrosine.

Experimental

Preparation of proteins for analysis

The skins were prepared by the method of Fraenkel & Rudall [1940] except where otherwise stated. The majority of them were very kindly provided by Dr Fraenkel. As described by Fraenkel & Rudall, part of the protein of the larval cuticle of *Sarcophaga* is extremely soluble in water and the bulk of it may be obtained by grinding the cuticle with sand in cold water for a short time. The whole of the protein is not removed by this treatment, however, and further yields can be obtained by soaking in water at 0° for several days, and following this by extraction with boiling water. The three fractions thus obtained are identical so far as total N and general physical and chemical properties are concerned. The protein is obtained more readily and without risk of degradation by extracting the skins at 50° with a buffer solution at *p*H 9·2, consisting of borax solution (19 g./l.), water, alcohol and ether in the proportions of 5 : 5 : 4 : 1. After 36 hr. no further material precipitable by trichloroacetic acid is extracted unless the skins remain in contact with the solvent for many weeks at room temperature. The borate extract is then filtered through paper pulp, the filtrate acidified with HCl to *p*H 3–4 and precipitated by 1/3 saturation with $(NH_4)_2SO_4$ or full saturation with NaCl. The precipitate is then centrifuged, resuspended in distilled water and dialysed against distilled water for 2 days. During such treatment the precipitate tends to go into solution; the mixture was therefore reduced *in vacuo*, transferred to a flat dish and kept in a vacuum desiccator over NaOH. The brittle translucent and almost colourless solid thus obtained was ground up with alcohol, extracted twice with boiling alcohol and then with boiling ether to remove fat. A similar product was prepared by a like treatment of the hawk moth cuticle. In the case of the lobster the integument was first finely ground in a mechanical mortar with the borate buffer mixture so as to give a soft mud.

The second protein was prepared from *Sarcophaga* cuticles by extracting the residues from the first with 5% NaOH at 50° for 5 hr. The extract was then filtered and the protein precipitated by adding several volumes of acetone. The fine precipitate was next suspended in distilled water and dialysed against

distilled water for 2 days. The procedure then followed that described above for the borate-soluble protein. Insufficient material from *Sarcophaga* was obtained for characterization by chemical analysis, but this was possible with the product derived from *Sphinx ligustri*.

Methods of analysis

The following techniques were used: Total N, micro-Kjeldahl; total S, micro-Carius (Weiler); total P, Fiske & Subbarow [1925]; total carbohydrate, Pirie's [1936] modification of Tillmans' & Philippi's method [1929]; cystine and methionine, Lugg's [1938, 2] modification of Baernstein's [1936] volatile iodide method; amino-sugar, Nilson [1936], Elson & Morgan [1933].

The N distribution in the proteins was determined by a modification of Thimann's [1926] method for the determination of the Hausmann number. Humin was neglected and accurate ammonia values were determined on separate hydrolysates of about 50 mg. protein. These were brought to *p*H 6–7 with NaOH, using bromothymol blue as indicator, transferred to a micro-Kjeldahl distillation apparatus and the ammonia distilled from an excess of borax solution into standard acid in the usual way. The ammonia-free hydrolysates for base determination were reduced to 10 ml., acidified and precipitated with phosphotungstic acid according to Thimann's procedure. The phosphotungstic acid was removed from both the base and monoamino-acid fractions by extraction with an amyl alcohol-ethyl alcohol-ether mixture according to Van Slyke [1915]. The removal of the phosphotungstic acid facilitated incineration for N determinations and comparison of the N distribution of edestin determined by this method with and without the removal of the phosphotungstic acid showed that a negligible quantity of N was lost during the extraction. The amino-N and hence the non-amino-N of the monoamino fraction was determined by the usual Van Slyke constant volume gasometric method. True amide-N values were determined by heating about 50 mg. protein with 5 ml. 2*N* HCl for 3 hr. on a boiling water bath and estimating the ammonia produced as in the previous determination.

Glycine was determined by Patton's [1935] colorimetric method, using 300–500 mg. protein instead of the 3 g. employed by that author, the amounts of reagents employed being reduced accordingly. Good values were obtained for duplicate analyses of gelatin (23·8 and 24·6%).

Tyrosine and tryptophan in the proteins of the whole cuticle were determined by Lugg's [1937] method, with minor modifications. The chitin residues after alkaline hydrolysis in the usual way were thoroughly washed with hot water and finally with dilute H_2SO_4 to remove the persistently adhering alkaline hydrolysate. The large bulk of washings and hydrolysate was then reduced to a small volume *in vacuo* and finally made up to 25 ml. The employment of Lugg's [1938, 1] alkaline stannite reducing reagent gave no marked difference in the tyrosine value obtained. The possible effect of chitin on the estimation of the two amino-acids was investigated by adding chitin to casein in the proportions in which it occurs in the cuticles under investigation. The effect was negligible as shown by Table 1.

Table 1. *The effect of chitin on the estimation of tyrosine and tryptophan*

Material	Chitin mg.	Protein mg.	Tyrosine %	Tryptophan %
Crab chitin	202	0·0	0·0	0·0
Insect chitin	181	0·0	0·0	0·0
Casein (N = 15·65%)	0·0	196	5·74	1·52
Casein + insect chitin	145	201	5·74	1·49
Casein + crab chitin	195	188	5·72	1·51

To determine sulphate the hydrolysates were adjusted and precipitated according to the standard methods of quantitative inorganic analysis. They were checked by precipitation with benzidine hydrochloride [Cole, 1933].

The proteins of the whole cuticle

Analytical data for the various intact Arthropod cuticles, the residues obtained from them by extraction for 30 hr. with borate buffer at 50°, and the residues obtained from these by further extraction with 5% NaOH for 5 hr. at 50°, are given in Tables 2–4. In all cases the borate buffer extracted a protein

Table 2. *Analysis of the intact cuticles of certain Arthropods*

Material	Total N %	Total S %	Total P %	Total carbohydrate as glucose %	Ash %
Sarcophaga falculata (blowfly) larval cuticle	9·0	0·56	0·0	0·4	0·56
Calliphora erythrocephala (blowfly) larval cuticle	10·7	0·40	0·0	0·5	—
Phormia terra novae (blowfly) larval cuticle	11·3	0·32	0·0	0·5	—
Sarcophaga puparium	11·3	0·59	Trace	—	1·9
Sphinx ligustri (hawk moth) larval cuticle	10·9	0·30	Trace	0·8	1·5
Dixippus (stick insect) adult abdominal cuticle	11·8	0·21	0·0	0·9	—
Locust adult abdominal cuticle	11·8	0·34	0·0	0·6	—
Prawn cuticle (decalcified)	9·4	—	+ve	+ve	—

Table 3. *The composition of cuticle residues after extraction with borate buffer mixture*

Material	Total N %	Total S %	Total carbohydrate as glucose %	Ash %
Sarcophaga larval cuticle	7·8	0·25	0·9	0·6
Sphinx larval cuticle	7·9	0·38	1·0	1·8
Dixippus abdomen	10·5	0·20	0·9	—
Locust abdomen	11·8	0·33	0·6	—
Lobster residue	6·9	0·44	0·7	—

Table 4. *The composition of cuticle residues after extraction with 5% NaOH*

Material	Total N %	Total S %	Total carbohydrate as glucose %	Ash %
Sarcophaga larval cuticle	6·5	0·0	0·9	2·5
Sphinx larval cuticle	6·5	0·24	0·9	2·1

having general properties similar to the product obtained from *Sarcophaga* larval cuticle, though the amount obtained from *Dixippus* and locust was small. The reagent, however, has left a large proportion of the carbohydrate and of the S-containing component intact. The data given in Table 5 are almost identical with those obtained from the residues of both *Sarcophaga* larval cuticle and puparia after extraction with 5% NaOH for 2 days at 100°, or after prolonged

diaphanol treatment [Fraenkel & Rudall, 1940]. All the residues still contain about 0·8% of material giving the orcin reaction for carbohydrate, and this is not removed by more prolonged treatment with any of the reagents concerned.

The borate- and water-soluble proteins

The proteins of Sarcophaga. The observations of Fraenkel & Rudall [1940] on the general precipitation properties of this protein were confirmed and extended; in addition it was found that these properties were shared by all the borate- and water-soluble proteins prepared from other insects. The protein is extremely soluble in water, and is not coagulated by heat. It is precipitated from solution completely by 10% trichloroacetic acid and also at *p*H 3–4, but not at higher values, by one-third saturation with $(NH_4)_2SO_4$ and saturation with NaCl. Many volumes of alcohol and of acetone are required to precipitate it from either acid or alkaline solution. Wet trichloroacetic acid precipitates will gel in alcohol or acetone, but water gels similar to those of gelatin have not been observed.

Table 5. *Nitrogen distribution in the water-soluble proteins of the cuticle*

Nitrogen values given are in percentages of total protein-N.

Material	Total N % protein	Amide-N†	Ammonia-N‡	Base-N	Mono-amino-N	Non-amino-N
Edestin (Osborne & Harris)	—	10·1	—	31·6	57·7	—
Edestin (Thimann [1926])	18·4	—	11·1	28·8	58·7	—
Edestin (modified Thimann method)	18·4	—	10·3	29·8	58·3	—
Sarcophaga protein	15·0	5·3	6·2	23·0	72·4	4·3
	—	—	6·2	26·4	70·0	3·5
Sphinx protein	15·1	7·4	8·5	18·0	75·0	—
	—	7·4	8·5	21·0	68·2	—
Lobster protein	15·4	8·5	9·5	18·1	70·4	—
Sarcophaga larval cuticle*	—	—	—	20·5	75·2	2·0
	—	—	—	19·3	77·8	3·0
Sarcophaga puparium*	—	—	—	22·7	67·0	3·5
Sericin A (Ito & Komori [1939])	17·37	—	12·14	16·91	70·10	0·61
Sericin B (Ito & Komori [1939])	16·88	—	12·22	18·20	69·19	1·16

* 5% HCl hydrolysis for 48 hr. to separate chitin, chitin removed and values calculated on percentage of total N of non-chitin fraction.
† Ammonia given after hydrolysis with 2*N* HCl for 3 hr.
‡ Ammonia given after hydrolysis with 20% HCl for 24 hr.

Table 6. *Some amino-acids and other components in the water-soluble cuticle proteins and in sericin*

Protein	Tyrosine %	Trypto-phan %	Glycine %	Hexos-amine as glucos-amine %	Carbo-hydrate as glucose %	Total S %	Total P %	Ash %
Sarcophaga protein	5·2	1·4	0·67	0·0	0·0	0·0	0·0	0·4
Sphinx protein prep. 1	11·7	0·5	1·32	0·0	0·0	0·87	0·0	0·2
Sphinx protein prep. 2	11·6	0·5	—	0·0	0·0	0·34	0·0	0·2
Lobster protein fraction 1	6·0	+ve	—	0·0	+ve	+ve	0·0	—
Sericin A				0·45§	0·37§			
	6·0*	1·0*	1·2†			1·2*		
Sericin B			1·5‡	1·08§	1·25§			

* Alders [1927].
† Abderhalden & Worms [1909].
‡ Strauch [1909].
§ Ito & Komori [1939].

Chemically the protein exhibits weak biuret and xanthoproteic reactions similar to those given by gelatin. Tests for carbohydrate, P, S, and for amino-sugars by the methods of Elson & Morgan [1933] and Nilson [1936] were negative. The product obtained by ice-cold water extraction of the living skin gives the same reactions; it is unlikely therefore to be the degradation product of a coagulable protein, though certain of its physical properties and the absence of S do, it is true, suggest a similarity to gelatin. The analytical data given in Tables 5 and 6 however do not bear out such a relationship; the glycine content is low, as also must be the proline content (non-amino-N, Table 5). The data are, indeed, more in keeping with the suggestion that the Arthropod cuticular proteins are akin to sericin (silk gelatin), but again the analogy must not be pressed too far, for they do not contain the very high proportion of serine that is characteristic of the latter protein. The data for threonine-N, and for β-hydroxy-α-amino-N given in Table 7 were obtained by the indirect method of Martin & Synge [1941]. The difference between the two values for any one protein probably

Table 7. *Minimum values for the distribution of nitrogen between α-amino-β-hydroxy-N and amide-N in some proteins*

Values given are in percentages of total protein-N.

Protein	Total N %	Amide-N	Total α-amino-β-OH-N	Threonine-N
Casein [Martin & Synge, 1941]	13·4	10·4	6·3	2·1
Casein	15·65	10·0	6·2	2·2
Sericin 1	17·1	10·6	21·4	4·4
Sericin 2	17·0	10·4	24·4*	1·9
Sarcophaga larval protein	15·0	6·9	6·8	1·8
Sphinx larval protein	15·1	7·8	6·5	2·5

* Allowing the reaction and aeration to continue for another hour this value was increased to 28·7% although continued aeration for the same period in the case of the amide determination and the hydroxyamino-N of other proteins did not give this large increase.

represents serine-N, and the high content recorded for each of the two samples of sericin confirms the recent findings of Nicolet & Saidel [1941]. It should be noted that the products referred to as sericin 1 and 2 in Table 7 were not prepared in the same way as the A and B of Ito & Komori [1937]. 1, was more soluble in water than 2, whereas B was precipitated by acid at *p*H 4·1 and A by adding alcohol to the mother liquor.

The protein of the hawk moth, Sphinx ligustri

A protein similar in physical and certain chemical properties to that of *Sarcophaga* was obtained from the larval cuticle of this insect. It exhibited certain marked and also certain less pronounced differences however; the tyrosine content, for instance, was extremely high. Two products made from different broods of larvae gave identical analytical data with the exception of total S, and the variations here may be due to the method of preparation. In the first the protein was precipitated from the borate extract by one-third saturation with $(NH_4)_2SO_4$ at *p*H 3–4: in the second the reagents employed were S-free and the precipitation was carried out at *p*H 3–4 with saturated NaCl. The high S content of preparation 1 therefore may have been due to retention of sulphate ions though one would have expected these to have been removed by the subsequent dialysis. Whether or not the S-containing component occurs in easily dissociable form is not known, nor is there as yet any indication of the

type of compound concerned. Experiments have shown that it contains organic S, and that this is not in the form of ethereal sulphate, cysteine, cystine, sulphydryl or methionine. The sulphur reactions for cystine, and Fink's [1927] test for sulphydryl groups, for instance, were negative for both the isolated protein and the intact skin; likewise a distribution of S by Lugg's [1938, 2] modification of Baernstein's [1936] volatile iodide method gave a negative result in both cases. The absence of inorganic S was shown in the following way. Duplicate dried samples of preparation 2 were dissolved by warming with a little distilled water and made up to about 50 ml. in *N* HCl: 2 ml. of a solution containing 36 mg. $BaCl_2, 2H_2O$/ml. were added and the whole kept. After 36 hr. the precipitate was centrifuged and the protein solution decanted. The precipitate was washed with hot water, transferred to a crucible and ignited. There was no appreciable ash, indicating the absence of free inorganic sulphate from the protein preparation. Duplicate hydrolyses each with about 500 mg. of protein were made by boiling it with 15 ml. 5*N* HCl for 12 hr. One hydrolysate was precipitated with $BaCl_2$ under standard conditions; no appreciable residue remained after ignition. The second hydrolysate was freed from excess HCl and treated with benzidine hydrochloride, but no precipitate was obtained. These experiments suggest that ethereal sulphate is not present in the protein. Negative results were also obtained from the intact cuticle of *Sarcophaga*.

The protein of lobster cuticle is similar to that of the two insects, the tyrosine content being comparable with that of *Sarcophaga*. Glycine was not determined.

The protein fraction soluble in warm alkali

Protein from the hawk moth. The original protein preparation effervesced when treated with acid; it was therefore redissolved in water and dialysed against distilled water. The dried preparation contained 11% ash, which was completely soluble in water and strongly alkaline. It was probably Na_2CO_3 derived from the alkali used for extraction. On the ash-free basis the preparation analyses as follows: total N, 12·4%; total S, 0·57%; carbohydrate (as glucose), 3·2%. The carbohydrate content of this fraction is higher than that of any other, showing that in the cuticle the carbohydrate is present either directly combined with the protein or in the form of polysaccharide. Tests for amino-sugars [Nilson, 1936] were negative. The sulphur values indicate that the S-containing component is more closely associated with the carbohydrate-rich fraction than with any other.

Protein from Sarcophaga *puparium*

It has been shown by Fraenkel & Rudall [1940] that a certain amount of the puparium protein may be extracted with cold water. This was confirmed by the fall in the total N from 11·0 to 9·3% after grinding with distilled water in a mechanical mortar for about 1 hr. As described below, all attempts to obtain the remaining protein in an intact state were unsuccessful, suggesting that the protein and chitin are not chemically separable entities, but are firmly bound by linkages at least as strong as those between the amino-acids of the protein.

Ammoniacal copper solutions, ethylene glycol, dioxan, anhydrous formamide, anhydrous formamide containing a little formic acid, saturated urea and strong cyanide solutions were found to have little or no solvent action upon intact powdered puparia. Acid solutions, even conc. HCl, had only a slow solvent action upon the residual water-extracted puparia compared with their action upon the soft larval cuticle of the same animal. Their action is not noticeably increased by the presence of reducing agents such as SO_2. Alkalis have much

more effect, but even when dilute (0·5*N* NaOH) have a strong disrupting action upon the protein. Here, again, reducing agents such as $SnCl_2$ do not promote solution.

Clark & Smith [1936] showed that pure chitin is readily dispersed at 95° in aqueous LiCNS previously saturated at 60°. Von Weinarm [1912] showed that proteins like keratins and silk fibroin were not dispersed by this reagent until very high concentrations of LiCNS and a temperature of 170° had been reached. Experiments with the puparia showed them to be similar to the latter substances in their dispersal properties. The dispersed product was partially reprecipitable with acetone after the removal of LiCNS by dialysis. However, the precipitated product and that remaining in solution both had a total N of about 11%, the same as the untreated material; consequently it appears that no effective separation of the protein and chitin had been obtained.

No further attempts to separate the protein from the chitin were made, but work was continued on partial hydrolysates of the whole skin. 5% HCl and 5% NaOH remove the protein by hydrolysis on heating to 100° for 48 hr. whereas the chitin is unaffected and can be filtered off so as to permit the hydrolysis of the protein being taken to completion in the usual way. Chitin residues obtained by this treatment contained 6·3–6·5% N (purified chitin 6·9% N) and are comparable with products obtained by much milder treatment. As shown in Table 5 a fully hydrolysed 5% HCl extract of *Sarcophaga* puparia has a N distribution comparable with that of a similar extract from the larval cuticle and of the protein isolated from it by borate.

Chemical changes on pupation

There was a decrease in the amount of tyrosine from 3·5 to 2·0% in the cuticles of a brood of *Sarcophaga* on pupation. Previous experiments had shown that chitin has no effect on the estimation of the tyrosine in casein; consequently it is unlikely that it had any upon the determinations with the cuticle protein. A comparison of prepupae and pupae from the same brood shows that there is a corresponding decrease in the total tyrosine in the whole organism, from 1·70 to 0·87%.

There is little doubt that much of the lost tyrosine, part of which must have come from the protein in the cuticle, has been oxidized to form pigmented products. That the oxidation has proceeded as far as the melanin stage is shown by the fact that the tyrosine value for the puparia is not increased if the extraction is made with alkaline stannite [Lugg, 1938, 2]. The stannite reduces the whole of the dark brown pigments, giving a clear, golden-yellow hydrolysate and an almost colourless chitin residue. On exposure to the air the hydrolysate rapidly resumes a dark brown colour indistinguishable from that of the normal alkali-hydrolysed puparia, showing that the pigment (melanin) is an oxidation product of a precursor held reduced by the action of the stannite. This oxidation is retarded but not completely inhibited if the reduced solution is acidified. Ether will extract from the reduced and acidified solution a small proportion of this easily oxidized material, which is in turn extracted from the ether by an alkaline solution, and in the latter state gives Arnow's [1937] characteristic reaction for dihydric phenols. The bulk of the reduced pigment remains in the original aqueous acid phase, from which it may be sharply and completely separated by extraction with amyl alcohol. Butyl alcohol was also found to have similar powers of extraction although amyl alcohol gave a better separation. The fresh amyl alcohol extracts are red but gradually turn brown on contact with the air. No reduced pigment can be extracted directly from the alkaline stannite hydro-

lysate and if the amyl alcohol extract from the acidified solution is removed and treated with alkaline solutions the colour changes to a light olive-brown and the pigment passes into the aqueous phase once more. The brown pigment extracted by acid hydrolysis of the puparia is also insoluble in amyl alcohol. Mazza & Stolfi [1931] extracted from the skin of the marine worm *Halla parthenopea* a red pigment, hallachrome, which was reversibly oxidized and reduced (see also Friedheim [1933]) and had similar amyl alcohol solubility properties to the above reduced pigments. They showed that it was the 5:6-quinone of dihydroindole-2-carboxylic acid, previously shown by Raper [1926] to be the red intermediate in the oxidation of tyrosine to melanin under the action of tyrosinase. Schmalfuss and co-workers [1927; 1933; 1935; 1937] have isolated 'dopa' and 3:4-dihydroxyphenylacetic acid from the hard, dark cuticles of certain insects. The above evidence suggests that a small proportion of the tyrosine derivatives in the puparium may be in the form of such dihydric phenols but that the majority of them are probably indole derivatives similar to hallachrome. Further, it is likely that they are formed by polyphenol oxidase in the cuticle, for Bhagvat & Richter [1938] have shown that the enzyme is present in high activity in the unmelanized skins of certain insects.

A few further experiments were made on the amino-acid content of the puparium protein. 3·4 g. of puparia were boiled under reflux with 20 ml. of 5*N* HCl for 24 hr. and cooled; the residual chitin was filtered off and washed with water. The filtrate and washings were reduced to a small volume, made up to 20 ml. with 20% HCl and boiled under reflux for a further 24 hr. Excess HCl was then removed by evaporation in the usual way and the dicarboxylic acids precipitated according to Foreman [1914]. The residual lime carried down the melanin pigments and the humin, and clean extracts were obtained, from both the dicarboxylic acid precipitate and the filtrate, on removing the Ca^{++} as oxalate. From the former 52 mg. of glutamic acid hydrochloride (N = 7·62%) and 36 mg. copper aspartate ($C_4H_5O_4NCu$, $4{\cdot}5H_2O$ requires H_2O, 29·2%; the anhydrous salt N, 7·2%: found 29·2% and 7·1% respectively). Repeated esterification of the clarified Foreman filtrate and saturation with gaseous HCl at low temperature failed to give any glycine ester hydrochloride. From the experience of Fischer [1902] and later workers it may be concluded that the puparium protein does not contain more than a few per cent of glycine.

Discussion

It will be seen that the amino-acid analyses of various Arthropod cuticles, though somewhat limited in scope on account of shortage of material, have shown that the constituent proteins differ from the collagens such as gelatin and are in certain respects akin to sericin. There is, for instance, general agreement in the values for amide-N, basic N, non-amino-N, tyrosine and tryptophan, and in the absence of cystine and methionine. On the other hand there is a sharp differentiation in the high serine content of sericin.

More interesting perhaps is the analogy that can be drawn between the insect cuticle and the sericin-fibroin complex of raw silk. The micro-crystalline skeletons of the two complexes are crystallographically comparable in some respects. The fibroin skeleton of silk differs from the majority of proteins in the predominance of the simple amino-acids glycine and alanine (glycine 43·8%, alanine 26·4% [Bergmann & Niemann, 1937]) and as Meyer & Mark [1930] have indicated, it is also structurally anomalous among protein fibres in that its X-ray diffraction photographs may be interpreted directly on the assumption that the chain is

composed of alternating glycine and alanine residues. In this sense fibroin is the only protein directly comparable with cellulose and chitin.

Silk is secreted by glands derived from the epidermal layer, as are the cells of the hypodermis which secretes the cuticle. Foa [1912] has shown that unsecreted silk in the seripterium of the silk-worm larva is a homogeneous substance soluble in boiling water, 5–20% NaCl and 5–10% Na_2CO_3, non-dialysable and non-heat-coagulable. Silk therefore must be formed on spinning by crystallization of fibroin within a sericin matrix. Ramsden [1938] has suggested a mechanism for this process. It is possible that chitin crystallizes in a similar way from a homogeneous fluid polysaccharide-protein complex secreted by the hypodermis, in other words, that the cuticle is preceded by a stage comparable with the known mucopolysaccharide-protein salts.

According to Meyer [1938] the mucopolysaccharide of synovial fluid may be spun to produce a material comparable with chitin in some of its physical properties. It is composed of long, doubly refracting fibres of considerable tensile strength. Alsberg & Hedblom [1909], moreover, have shown that chitin may be redispersed after prolonged treatment with dilute HCl in the cold to give a water-soluble form comparable with a soluble mucopolysaccharide. A further similarity between the vertebrate tissues in which mucoproteins occur and the chitin-protein complexes of invertebrates is the fact that they are both selectively calcified.

Summary

Protein, carbohydrate and a sulphur-containing component have been shown to be present in the cuticles of a variety of insects.

The protein fractions are usually large and when extracted show similar general properties.

The protein of the larval cuticle of *Sarcophaga falculata* was characterized by the determination of its nitrogen distribution, tyrosine, tryptophan, glycine and β-hydroxy-α-amino-acid values. Sulphur and conjugated non-protein components were absent. The data showed that the protein was quite distinct from collagen in its chemical composition and that it bears a close resemblance to sericin, although the characteristically high serine content of the latter was not recorded for the cuticle protein.

Similar proteins from the hawk moth larva *Sphinx ligustri* and the lobster were characterized in the same way. Certain marked differences from the blow-fly protein were recorded in each case. The tyrosine value of 11·7% of the weight in the case of the hawk moth protein is remarkably high.

The protein of the hard, melanized puparium of *Sarcophaga* appeared to differ from the larval protein mainly in a loss of solubility and the replacement of a proportion of its tyrosine by melanoid oxidation products. These latter were reduced, extracted and shown to have some of the properties of hallachrome.

The possible formation of chitin from a homogeneous fluid polysaccharide-protein complex secreted by the hypodermis is briefly discussed.

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124. PROTEIN METABOLISM IN SEED GERMINATION

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Few aspects of plant physiology have excited an interest comparable with that manifested for many decades in the protein metabolism of germination. A modern interpretation of the earlier work and a correlation of it with recent research have been given by Chibnall [1939]. Our knowledge of the changes in protein composition which accompany germination still, however, largely lacks the support of direct experiment.

In recent years claims have been renewed that germinated seeds, or seedlings, are frequently of greater value in stock feeding than the ungerminated seeds; see, for example, the review by Leitch [1939]. In regard to palatability, digestibility, vitamin content, biological value of the protein etc., these claims may well be justified in some instances. It has long been known, however, that with some seeds an enormous loss of protein N and corresponding gain of non-protein N, accompany the transition from seed to seedling. Thus seeds containing much protein and but little fat or carbohydrate are of this type, as Schulze [1880] and others have shown, and a large fraction of the non-protein N produced usually occurs in asparagine. In the event of the total seedling protein being of higher biological value than that of the seed, the question remains whether there has been a net gain or loss of those amino-acids, contained in the proteins, which are dietary essentials. The non-protein N substances produced must be accorded nutritive value, particularly if they should happen to consist not so much of the amides, asparagine and glutamine, but of a relatively unchanged assemblage of amino-acids split from the seed protein by zymolysis.

In this article data will be presented giving direct information on some of the points raised above. The work was done with the blue lupin, *Lupinus angustifolius*, seedlings of which were being examined as a source of asparagine. Seedlings of the yellow lupin, *L. luteus*, might have been a better source, for as Schulze [1898] showed, the seeds of this plant have a lower carbohydrate plus fat to protein ratio than seeds of *L. angustifolius*. The latter were used because they were available in quantity.

Experimental

Preparation of materials—N balance

The seeds, which showed about 96% viability, were germinated on wet, purified cotton wool, and the seedlings were cultured on water in virtual darkness at about 27°. 14 days after setting the seeds, that is, about 12 days after the emergence of the rootlets (a few emerged in the first day and a few late ones in the third day), the crop from 110 germinated seeds was harvested.

The testas were discarded and the seedlings (*c*) were divided into cotyledons (*d*) and axial organs (*e*), and these latter in turn were divided into roots, or the portions of the hypocotyls which had been submerged in the water (*g*), and stalks with their young plumules (*f*). Another 110 ungerminated seeds taken at random were divided into testas (*a*) and embryos (*b*); see Table 1.

Table 1. *Germination of 110 seeds of* Lupinus angustifolius

Fresh wt. 26·55 g., dry wt. 23·78 g. Seedlings harvested 1. viii. 1940.

	(*a*) Testas	(*b*) Embryos	(*c*) =(*d*)+(*e*) Seedlings	(*d*) Coty-ledons	(*e*) =(*f*)+(*g*) Axial organs	(*f*) Stalks + plumules	(*g*) Roots	(*c*)–(*b*) Net gain
Fresh wt. (g.)	6·60	19·95	209·1	44·7	164·4	143·6	20·8	189·1
Dry wt. (g.)	6·10	17·68	14·70	5·22	9·48	8·13	1·35	−2·98
(% N, on dry wt.)	(0·80)	(7·12)	(8·56)	(6·61)	(9·63)	(10·04)	(7·18)	—
Total N (g.)	0·049	1·258	1·258	0·345	0·913	0·816	0·097	0·000
Coagulable N (g.)	—	1·095	0·279	0·139	0·140	0·105	0·035	−0·816
Protein N (g.)	—	0·976	0·259	0·132	0·127	0·094	0·033	−0·717
Dry wt. protein prep. (g.)	—	10·95	—	2·30	3·21*	2·53	0·68	—
(% N in dry protein prep.)	—	(8·91)	—	(5·74)	(3·96)	(3·70)	(4·88)	—

* By addition of (*g*) and (*f*).

Samples of these materials were taken for moisture and total N estimations. The main bulks (except testas (*a*) on which no further work was done) were treated as described in other articles [Lugg, 1938, 2, 3] for removal of non-coagulable and other non-protein nitrogenous substances, by macerating, and extracting with hot water at *p*H 4·5 (the residue at this stage containing 'coagulable N'), followed by alcohol, dilute Na citrate solution at *p*H 3·5 and ether (the residue at this stage containing only 'protein N'). As the initial coagulation was not assisted by addition of alcohol, several % of the protein N of (*f*), (*g*) and (*d*) may have gone into the non-coagulable N fractions [Lugg, 1939]. The dried materials remaining after extraction constituted crude protein preparations. All N estimations were made by the Kjeldahl method. The data are shown in Table 1. The main bulks of the crude protein preparations were kept in reserve after those from (*g*) and (*f*) had been mixed in the proportion necessary to represent the axial organs (*e*). The analyses of these preparations are described later.

The total N in the 110 seedlings was 2·8 % lower than that in the 110 ungerminated embryos. There was no visually obvious correlation between size of seed and viability, and in any case viability was high. The probability of a 2·8 % or greater difference in total N content between any two groups of 110 seeds taken at random, was calculated as 0·36 from a statistical analysis of data for ten such groups. It has been assumed that there was neither assimilation of atmospheric N, transfer of N to or from the testas nor discharge of seedling N into the water. The lower N content of the seedlings has been attributed solely to random sampling errors, and the corresponding corrections have been made in Table 1 to show no net gain or loss of total N.

From the non-coagulable material in the stalks and plumules (0·711 g. N), 2·56 g. of recrystallized asparagine monohydrate (0·477 g. N) were obtained.

Partial analyses of the protein preparations

The amide, tyrosine, tryptophan, cystine plus cysteine and methionine contents of the protein preparations from ungerminated embryos (*b*) and from the cotyledons (*d*) and axial organs (*e*) of the seedlings, were estimated.

The methods used, estimation of NH_3 liberated by mild hydrolysis with acid for amide; colorimetric estimations after alkali-stannite hydrolysis for tyrosine (diiodotyrosine and dibromotyrosine were presumed to be absent from the preparations) and tryptophan, and sulphate estimations following differential oxidation for cystine plus cysteine and methionine, have been described in detail in other articles [Lugg, 1937; 1938, 1, 2, 3] wherein their applicability to crude protein preparations has been demonstrated.

Some difficulty was encountered in the tryptophan estimations, as the tryptophan contents were rather small and there were fairly large corrections for adventitious coloration. Incidentally it is best, when concentration of a prepared acidified hydrolysate before mercuration is desirable, to render the hydrolysate alkaline and evaporate at fairly low temperature under reduced pressure. But this entails increase in the ultimate sulphate content, and it has been found satisfactory in certain cases (some leaf-protein preparations) to distil water from the acidified hydrolysates at temperatures up to 45° [Lugg, 1938, 1]. It is safer to evaporate at temperatures up to 25°, and this is conveniently done by exposing at low pressure over H_2SO_4 in a vacuum desiccator. At the higher temperature there was evidence of loss of tryptophan in the case of the lupin embryo protein preparation, due presumably to production of aldehydes and their condensation with some of the tryptophan.

In Table 2 are recorded the partial compositions of the protein preparations. Corrections have been made for such systematic errors as are revealed when the pure amino-acids are put through the analytical processes. For discussion of

Table 2. *Partial compositions of the proteins*

Protein prep.	% N in prep.	% Amide N	% Tyr. N	% Try. N	% Cyst. N	% Meth. N	% Cyst. + meth. N	SO_4-S/N ×100	% Ash in prep.
(*b*) Embryos	8·91	9·82	1·70	0·62	1·16	0·24	1·40	0·002	1·1
(*d*) Cotyledons	5·74	7·43	2·21	0·79	1·43	0·59	2·02	0·007	1·5
(*e*) Axial organs	3·96	5·35	2·24	0·91	1·09	1·03	2·12	0·15	1·7

other possible systematic errors (assumed to be zero in the forthcoming discussion) reference may be made to the articles describing the methods. The cystine plus cysteine and methionine estimations have been based upon the assumption that all the S in the protein preparations may be accounted for in these amino-acids and in sulphate (either inorganic or ethereal). The random errors of estimation are believed not to exceed the amounts shown in parenthesis in the following description of the contents of the Table. In the order of the columns are: the label of the preparation, the percentage of N in the dry preparation (0·5 %, affecting the other errors), the amide content (1·5 %), tyrosine content (2 %), tryptophan content (5 %), cystine plus cysteine content (2 %), methionine content (4 % for (*e*), 6 % for (*d*) and 8 % for (*b*)), the sum of the last two values (2 %), sulphate-S as a percentage of the weight of N in the preparation (10 % for (*d*) to 100 % for (*b*)), and finally the percentage of ash in the preparation after ignition in air at about 600°. The amino-acid and amide contents have been reported on a N basis, that is, as percentages of the protein N occurring in these forms. They are mostly the means of duplicate or triplicate estimations, and some of these had to be made on a smaller scale than usual because only small amounts of material were available. Values in the last two columns are to be taken as measures of various impurities.

Discussion

As Table 1 shows, there was a loss of 73·5 % (0·717 g.) of protein N during the transition of the lupin embryos to 14-day seedlings. But the gain in non-coagulable N was 0·816 g. The difference between the coagulable N and protein N of the embryos (0·119 g.) is probably to be attributed to the presence of considerable lipin. It is to be noted that to an even greater extent than the protein N, this was converted into non-coagulable forms by germination.

No attempt was made to obtain asparagine from the cotyledons or roots. The amount separated in recrystallized form from the non-coagulable material in the stalks with plumules, represented 67 % of the N in that material, 49 % of the non-coagulable N in the entire seedlings and 38 % of the total N in the seedlings or in the embryos.

Some points of considerable interest emerge from Table 2. It will be noticed that the protein of the embryos had a fairly low tryptophan content and a distinctly low methionine content. Osborne & Campbell [1897] found that the various proteins in the embryos of *L. angustifolius* all contained about 18 % of N when carefully purified, and if it be assumed that the mixed embryo proteins in a pure state contained 18·0 % N, a simple calculation gives 0·81 % tryptophan and 0·46 % methionine in the embryo protein on a weight basis.

From the standpoint of animal nutritional requirements, it seems from the work of Bauguess & Berg [1934] that a tryptophan content no higher than this might render the lupin embryo protein somewhat unsatisfactory at a low level of intake. And from the work of White & Beach [1937–8], who found the very poor value of arachin as a rat food to be associated with its low methionine content (0·54 % according to Baernstein [1932]), and that of Womack *et al.* [1937], it must be concluded that in spite of a moderately high cystine content the low methionine content renders the lupin embryo protein very unsatisfactory.

The protein of the axial organs appears, from these analyses, to have been approaching in composition those of the photosynthesizing leaves of leguminous plants, and it is possible that the composition of the protein of the etiolated plumules themselves may have corresponded quite closely with that of the protein of photosynthesizing leaves of this plant. For the plumules were small, and although they were presumably relatively rich in protein (Schulze & Castoro [1903] found them to be so in the case of *Lupinus albus*), a considerable part of the protein in the axial organs must have been present in the stalks and roots (26 % is known to have been present in the roots) and there is at least meagre evidence that the protein in petioles may not correspond extremely closely in composition with that in the leaves they support [Lugg, 1940].

Recent work on the whole leaf proteins of legumes [Lugg, 1940, and unpublished data] suggests that the earlier analyses of samples of protein extracted from legume leaves [Chibnall, 1939; Lugg, 1938, 3] would be reasonably valid for the whole protein of those leaves. It was pointed out that many of the tryptophan estimations in the earlier analyses [Lugg, 1938, 3] were vitiated by the presence of interfering indoles. This difficulty has not been encountered in the more recent work. On the basis of what is now known of the composition of the leaf proteins of legumes generally, the protein of the lupin axial organs contained about 100 % of the amide N which might be expected in the protein of the green leaves of this plant, about 90 % of the tyrosine N, about 60 % of the tryptophan N, about 90 % of the cystine plus cysteine N and about 80 % of the methionine N.

The composition of the protein of the cotyledons suggests that it too was approaching the composition which might be expected of the protein in the

green leaves of this plant. The amide content was high whereas the tryptophan and methionine contents were both low, and the impression is gained that the cotyledon protein was something of a compromise between those of the embryos and axial organs in composition.

When seedlings with epigeal cotyledons are grown in the light, the cotyledons, like the plumules, become green and may be presumed to have a photosynthesizing function. They might therefore be expected to contain proteins characteristic of the normal photosynthesizing tissues as well as reserve seed proteins not yet used up. The amide, tryptophan and methionine contents of the lupin cotyledon protein lie in such a position between those of the proteins of the embryos and axial organs, as to suggest that in terms of protein N, the cotyledons contained about as much axial organ as embryo protein. But the tyrosine content of the cotyledon protein was only doubtfully lower than that of the axial organ protein, and the cyst(e)ine content was actually somewhat higher than that of the protein of either the embryos or axial organs. Among possible explanations are (1) that the different proteins originally present in the cotyledons may have been used up at different rates, (2) that even if the rates were similar, the proteins may have been degraded stepwise and if cyst(e)ine residues, for example, were rather late in being removed, degraded protein relatively rich in cyst(e)ine could have resulted, and (3) that (1) and (2) may have operated together. But in regard to degraded proteins there is the unanswered question of the stage of degradation or synthesis at which the methods used in this experiment would permit such protein to enter the category of 'non-coagulable nitrogenous substances', and for this reason, if for no other, it is profitless to speculate further on the nature of the cotyledon protein.

It remains to discuss the gain or loss by germination of amide and amino-acid residues contained in protein. Table 3 has been drawn up for this purpose

Table 3. *Retention and regeneration of protein components in the proteins of the 110 seedlings*

	(*b*) Embryos	(*c*) =(*d*)+(*e*) Seedlings	(*d*) Cotyledons	(*e*) Axial organs	(*c*)−(*b*) Net gain	100 (*c*)/(*b*) % Retention + regeneration
Protein N (mg.)	976	259	132	127	−717	26·5
Amide N (mg.)	95·8	16·6	9·8	6·8	− 79·2	17·3
Tyrosine N (mg.)	16·6	5·8	2·92	2·84	− 10·8	35
Tryptophan N (mg.)	6·1	2·2	1·04	1·16	− 3·9	36
Cyst(e)ine N (mg.)	11·3	3·3	1·89	1·38	− 8·0	29
Methionine N (mg.)	2·3	2·1	0·78	1·31	− 0·2	90
Cyst. meth. N (mg.)	13·7	5·4	2·67	2·69	− 8·3	39

from the data in Tables 1 and 2, and gives, among other things, a comparison of the amounts of amide N and amino-acid N contained in the protein of the embryos with those contained in the proteins of the seedlings (by addition of the amounts contained in the proteins of the cotyledons and axial organs). The last but one column in Table 3 shows the net 'gains' resulting from germination. They are all negative. The last column shows the percentage retention plus regeneration of the protein components.

Whereas of the original protein N, 26·5 % could be accounted for in the seedlings, only 17·3 % of the amide N could be so accounted for, but more than 26·5 % of each of the other components. The most interesting case is that of methionine which, alone of the components, shows anything like complete retention plus regeneration. It is regrettable that the value shown (90 %) is

subject to random errors of the order of 10%. It is possible too that of the seedling protein several % may have escaped flocculation and coagulation, as mentioned earlier. Consequently the protein methionine balance cannot be very accurately known. The loss may have been somewhat greater than 10% or there may have been a gain of several % by synthesis. If the results be taken to indicate that the supply of original protein methionine may have been a limiting factor in the regeneration of seedling protein, then, as considerable cyst(e)ine was not used up in the regeneration, this would probably imply that the etiolated seedlings, unlike mature plants growing in the light, were unable to synthesize methionine.

There was scant provision for possibilities of this sort in the theories of protein regeneration advanced by the earlier workers. The inability of etiolated seedlings of this type to develop beyond a certain stage and the piling up of amides and other non-coagulable nitrogenous substances instead of regenerated protein, were attributed in the theories of Schulze [1898] and Prianischnikow [1899] to lack of suitable carbohydrates or other non-nitrogenous metabolites. It is true that Prianischnikow, in opposition to Schulze, considered the seed protein amino-acids to play a primary part and the amides a secondary one in protein regeneration, but in his theory the first limitation upon regeneration occurs when, by lack of other metabolites, the seed protein or amino-acids have to supply the seedlings' energy requirements. Chibnall [1939] insisted that Paech's [1935] hypothesis concerning the breakdown and synthesis of protein in plant tissues becomes more acceptable if the factor 'active carbohydrate' is replaced by 'α-keto-acids', and pointed out that even when ample carbohydrate and asparagine (as a source of N) are present, a failure at any of the steps in the reactions: sugar→α-keto-acid→α-amino-acid→protein, could limit protein regeneration. Thus, although Chibnall did not discuss the possibility of the regeneration being limited by the supply of specific seed-protein amino-acids, failure at some step in a chain of reactions similar to the one he described, but involving S as well as N, might limit the supply of methionine for protein regeneration to the amount existing in the seed protein. And if that amount were very small it could be a first limiting factor in protein regeneration.

No whole leaf proteins are known to be nutritionally deficient, and the lupin seedling protein was probably of much higher biological value than that of the embryos. But the amounts of most of the dietary essential amino-acids, contained in protein, were probably reduced by the transition from seed to seedling (64% of the tryptophan, for example, was missing) and it is highly improbable that the amounts lost in this way could have been fully present among the non-coagulable substances, because the asparagine production was very large and must have involved the metabolism of large quantities of amino-acids. For feeding purposes a greatly increased degree of protein regeneration would have been desirable. Such advantages as may exist in the feeding of seedlings instead of seeds would probably be increased, with seeds of this type, by culturing for much longer periods and in light instead of darkness.

Summary

Etiolated seedlings, in which more than 38% of the N was present as asparagine, were grown from seeds of the blue lupin, *Lupinus angustifolius*. The retained plus regenerated protein N represented only 26·5% of the protein N of the embryos. The proteins in the embryos and in the cotyledons and axial organs of the seedlings, contained respectively (on a N basis): 9·82, 7·43 and 5·35% amide N; 1·70, 2·21 and 2·24% tyrosine N; 0·62, 0·79 and 0·91%

tryptophan N; 1·16, 1·43 and 1·09% cyst(e)ine N; and 0·24, 0·59 and 1·03% methionine N.

From these analyses the axial organ protein appears to have been approaching in composition those of the leaves of legumes. The cotyledons probably contained protein of similar composition mixed with incompletely broken down embryo protein. In the protein of the seedlings, it was possible to account for 17·3, 35, 36, 29 and 90%, respectively, of the amide N, tyrosine N, tryptophan N, cyst(e)ine and methionine N, originally present in the embryo protein.

It has been pointed out that the embryo protein must be of low biological value on account of its very low methionine content, and that whereas the seedling protein was probably of much higher biological value, germination probably resulted in a net loss of most of those amino-acids, present in protein and among the non-protein nitrogenous substances, which are dietary essentials.

The possibility has been discussed of the low methionine content of the embryo protein imposing a limitation upon protein regeneration in the etiolated seedlings.

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125. STUDIES ON THE 'FERMENTATION' OF CEYLON TEA

3. RESPIRATION AND TEA FERMENTATION

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THE production of CO_2 during fermentation in the manufacture of black tea has been observed by previous workers [Evans, 1928, 1, 2; Bosscha & Brzeskowsky, 1916; Roberts, 1939]. Evans (Ceylon) found that tea leaf like most other plant materials had a respiratory quotient of 1·0 which, however, did not appreciably change during withering. While crushing had no effect on the R.Q. of fresh leaf it caused a marked decrease in the R.Q. of withered leaf, due to a very large increase in the O_2 uptake associated with oxidation of polyphenols. This O_2 uptake, which is initially very rapid, gradually falls off until the ratio CO_2/O_2 again approximates to unity. The volume of CO_2 produced during fermentation decreases only slightly with time, e.g. 40 g. of rolled leaf at 18°, which produced 4·6 ml. in the 1st hr., still formed 3·8 ml. in the 7th. As a result of these observations Evans [1928, 2] concluded that 'the respiratory process is upset when the withered leaf is crushed, and during fermentation the normal process of respiration is not observed'.

Bosscha & Brzeskowsky [1916] showed that CO_2 is developed during the fermentation of completely sterilized leaf and concluded that it is not a product of growth of micro-organisms.

In north-east India, Roberts [1939; 1940] has studied in greater detail the output of CO_2 by fermenting tea leaf and has shown that it arises from oxidation of carbohydrates (glucose) present in tea. Roberts advanced an explanation in which this reaction is an intermediate step in his mechanism of fermentation. *o*-Quinones produced by oxidation of polyphenols by the cytochrome-cytochrome oxidase system either form condensation products or bring about the oxidation of non-tannin substances (glucose) through cozymase and dehydrogenases. The resulting reduced cozymase reacts with oxidized cytochrome giving reduced cytochrome which once again takes part in the cycle of reactions. Oxidation of carbohydrate is thus shown to influence continuous polyphenol oxidation.

In formulating this mechanism of fermentation Roberts has attempted to establish a relationship between respiration and fermentation of tea leaf. In fact, he is of the opinion that fermentation of tea is only an uncompensated respiration. Extensive mechanical injury resulting in the dispersion of coenzymes has been stated to be responsible for the switch over from respiration of tea leaf to its fermentation.

Parallel investigations on Ceylon leaf have led to interesting differences of conception of the relationship between fermentation and respiration. The leaf tissue resulting from the rolling process, and also, to a different extent, minced leaf, is regarded as a heterogeneous mixture of damaged and undamaged tissue. It seems improbable that even the finest mincing will result in the disruption of

every single cell. This means that undamaged tissue will continue more or less normal respiratory activity, while in the case of exposed cell contents enzymic oxidation with atmospheric O_2 will take place at a comparatively rapid rate. The latter activity accounts for the observed rapid increase of O_2 uptake by damaged tea leaf.

In the investigations described below, mincing was effected by means of a chromium-plated domestic mincing machine with the finest available cutter. In this case there is no question of complete disruption of the organic structure of the leaf although there is undoubtedly more damage than in factory 'rolled' leaf.

Evans showed that after an initial increase of O_2 uptake when leaf is damaged, with corresponding reduction of the R.Q. there was a steady reduction of O_2 uptake per hr. until the R.Q. again approached unity and remained at that level for several hours. The obvious explanation is that normal respiratory activity continues after the oxidation of exposed polyphenols has ceased.

It was shown by *in vitro* studies [Lamb & Sreerangachar, 1940] that the main reaction in fermentation, namely the oxidation of polyphenols, was not attended by any CO_2 production. Thus it was concluded tentatively that the CO_2 output observed in the fermenting leaf did not have its origin in the fermentation reaction itself. The apparatus employed for manometric studies was not, however, capable of high accuracy. The work has now been repeated and extended by the use of the more accurate Barcroft apparatus[1] and the results are reported in the present communication.

Experimental

Technique. The *in vitro* study of the O_2 uptake during the oxidation of tea tannin by our enzyme preparations was conducted by placing the enzyme powder in pliofilm boats left floating over the substrate solution in the reaction flasks. Shaking effects the admixture of the enzyme and substrate whenever desired. For absorption of CO_2, KOH was added to the central tube in the flask, rolls of filter paper being employed to ensure an adequate surface for absorption. 2 ml. substrate solution (2·8 %) and 50 mg. enzyme preparation, obtained by acetone treatment of fresh leaf as described in our previous communication [Lamb & Sreerangachar, 1940], were usually taken for each experiment.

Observations on respiration were made by cutting leaf into a few fairly uniform pieces and suspending these pieces in distilled water. While working with leaf suspensions 200 mg. samples of leaf mince were usually taken. Mincing was done as rapidly and as finely as possible and the mince well mixed before sampling. The material was suspended in 2 ml. water and after closing the taps the apparatus was shaken at a speed of 120–140 oscillations per min. Comparatively very little oxidation occurs before commencement of readings and the differential principle of the Barcroft apparatus eliminates small temperature variations. Brodie's solution was employed as manometric fluid in order to obtain fairly large readings. Unless otherwise stated all experiments were carried out at 25° in a thermostat and O_2 absorption results recorded in μl./200 mg. fresh tissue weight, employing the modified formula of Dixon & Keilin [1933] for calculating the vessel constants. Agreement between replicates has been found to be very good and in the interpretation of results a consistent difference of 10 % was considered to be significant.

[1] Kindly lent by Mr M. Sreenivasaya, Indian Institute of Science, Bangalore.

Oxidation of pure theotannin by tea enzyme preparation

Theotannin was prepared according to the method of Shaw [1935] and after removal of free gallic acid by dry ether a concentrated solution (2·8 %) was obtained. 2 ml. of this solution were reacted upon by 50 mg. of enzyme powder preparation in one of the twin Barcroft vessels while the other contained suitable inactive control substances. The difference between the values of O_2 uptake with and without the use of KOH gave the amount of CO_2, after correcting for the variation in the vessel constants for CO_2 and O_2.

Table 1 records the results of two separate experiments which are typical of many others obtained in support of our previous conclusion that oxidation of pure tea tannin substrate is not accompanied by CO_2 output.

Table 1. *O_2 uptake during enzymic oxidation of amorphous tea tannin*

min. ...	5	10	20	30	60	75	90	105
I: With KOH, μl.	18	36	67	103	218	272	321	359
Without KOH, μl.	20	40	82	118	215	265	312	348
min. ...	10	20	40	65	80			
II: With KOH, μl.	22	42	120	192	241			
Without KOH, μl.	18	53	120	187	228			

The enzyme preparation contained both soluble and insoluble enzyme systems and when an attempt was made to estimate separately the activities of these, it was found that under the conditions of experiment the soluble enzyme was almost completely inactivated by the high tannin concentration. After the extraction of all soluble enzyme, the activity of the insoluble enzyme was only very slightly higher than the activity of the unextracted enzyme powder (Table 2).

Table 2

min. ...	10	22	45	60	75	90	105	120
Extracted enzyme, μl. O_2	51	87	136	158	187	219	241	261
Unextracted enzyme, μl. O_2	42	78	125	149	174	192	219	238

The insoluble enzyme must be employed in the wet condition as otherwise its activity may suffer by drying. In a particular experiment the wet extracted enzyme was again treated with acetone and quantities (39·6 mg.) corresponding to the original weight of the unextracted enzyme (50 mg.) when employed on the same quantity of substrate showed about 40 % decrease in the uptake of O_2.

Reactions in tissue suspensions

As observed by Evans [1928, 1] and more recently by Deb & Roberts [1940], the R.Q. of respiring tea leaf is very nearly 1·0 and so very little volume change is recorded if no KOH is employed in the Barcroft vessels. But when the leaf is mechanically damaged, oxidation of polyphenol occurs in the damaged cells while in the undamaged cells the respiration continues: the R.Q. therefore falls enormously. Tables 3–6 show the values of R.Q. obtained for fresh and withered leaf before and after mincing, results being recorded in μl. gas per 200 mg. tissue.

The change in the R.Q. on mechanical injury to the leaf can be explained as due to two principal causes, viz.: (1) high O_2 uptake required for polyphenol oxidation, and (2) reduction in the respiring leaf surface resulting in a lower CO_2 output. Of these causes the significance of the reduction in amount of respiratory tissue has not hitherto been recognized.

If, however, such a hypothesis is correct then there should be some direct relation between the fall in R.Q. and the extent of crushing or mechanical

Table 3. *Respiration of fresh leaf*

min. ...	15	30	45	60	75	90	105	120
O_2 uptake, μl.	19	44	67	97	130	160	193	218
CO_2 output, μl.	22	49	71	98	125	152	178	201
R.Q.	1·20	1·11	1·06	1·00	0·93	0·95	0·92	0·92

Table 4. *Respiration of withered leaf*

min. ...	15	30	45	60	75	90	105	120
O_2 uptake, μl.	14	26	37	46	56	70	88	97
CO_2 output, μl.	13	27	38	49	58	69	80	89
R.Q.	0·96	1·05	1·02	1·06	1·04	0·99	0·91	0·91

Table 5. R.Q. *of fresh leaf mince*

min. ...	15	30	45	60	75	90
O_2 uptake, μl.	60	105	138	156	165	174
CO_2 output, μl.	19	28	46	53	53	56
R.Q.	0·31	0·27	0·34	0·34	0·32	0·32

Table 6. R.Q. *of withered leaf mince*

min. ...	15	30	45	60	75	90
O_2 uptake, μl.	40	76	112	147	185	225
CO_2 output, μl.	9	12	14	23	39	63
R.Q.	0·23	0·16	0·12	0·16	0·21	0·28

injury to the leaf. To put this to the test, two representative samples of leaf were taken: one part was minced and 200 mg. mince weighed out; of the other part 200 mg. leaf were first weighed and then ground with sand in a pestle and mortar. The O_2 uptake and CO_2 outputs of these samples when suspended in 2 ml. water were manometrically measured. Table 7 displays these results while Table 8 gives the changes in R.Q. for the 1st half hr. and the initial rate of fermentation ($-Q_{O_2}$) resulting from different degrees of tissue damage.

Table 7

min. ...	10	20	30	40	50	60	90
Total O_2 uptake							
Minced leaf, μl.	40	76	103	127	145	154	174
Ground leaf, μl.	69	147	169	183	192	196	210
CO_2 output							
Minced leaf, μl.	14	26	35	46	49	53	60
Ground leaf, μl.	10	19	30	37	44	46	51

Table 8

	R.Q.	$-Q_{O_2}$
Whole leaf	1·0	2·0
Minced leaf	0·34	6·0
Ground leaf	0·18	11·4

It may be concluded from the above results that a more intense mechanical damage is accompanied by a higher O_2 uptake and a lower CO_2 output. The ratio of damaged to undamaged cells would determine the actual extent of variations. Deb & Roberts [1940] have observed a similar effect of mechanical injury on the anaerobic fermentation[1] in which the greater the extent of mechanical damage the lower the rate of anaerobic fermentation.

[1] This is to be distinguished from 'tea fermentation', which is an entirely different, aerobic process.

The reactions in the damaged tissue have been explained by Roberts [1939] on the coenzyme dispersion theory. The decrease of carbohydrate oxidation or the production of CO_2 results from a dilution of coenzymes necessary for the process and more of the *o*-quinone produced by earlier reactions undergoes condensation. On suspension of the leaf mince in water, there will be a further dilution of coenzymes with similar effects as above on the carbohydrate oxidation and the accumulation of polyphenol oxidation products. It is possible, however, to explain this latter observation also by the respiration surface reduction theory. At higher dilutions the fine mince will be in a state of submersion under water and the leaf surface having access to air will be greatly diminished. Oxidation of polyphenol itself will be very little affected by this but the production of CO_2 will be markedly reduced.

In an attempt to correlate the results of manometric measurements of minced leaf suspensions with those of factory fermentation, Roberts has ignored the effect of different degrees of crushing on the rate of fermentation. Under the factory conditions, the rolling process does not bring about the same order of mechanical injury to the tissue as in fine mincing and it is highly probable that this difference may also be a factor in accounting for the much slower rate of fermentation in the factory.

Separation of fermentation and respiration reactions in the minced tea leaf

In order to investigate the origin of CO_2 production in the fermenting leaf it is necessary to separate the two coexisting processes namely fermentation and respiration, and the two methods employed for this purpose are described below.

(1) *Washing of minced leaf.* If the minced tissue is quickly washed free of the adhering juice and the contents of the damaged cells, fermentation is almost completely inhibited owing to lack of the polyphenolic substrate. This is shown by the fact that on omission of KOH in the Barcroft vessels, there is scarcely any registered O_2 uptake by the washed tissues which remain predominantly green even after 4 hr. The R.Q. is 1·0 and respiration, which is confined to the undamaged cells, is unaffected by washing. In Table 9 are given results of two typical experiments which show that both the washed and unwashed tissues produce almost the same amount of CO_2.

Table 9. *Effect of washing on CO_2 output*

min. ...	10	20	30	40	50	60	70	80	90
I: Washed tissue, μl. CO_2	14	26	33	42	51	56	63	70	76
Unwashed tissue, μl. CO_2	14	19	30	46	49	51	55	56	60
II: Washed tissue, μl. CO_2	10	19	26	30	37	46	53		
Unwashed tissue, μl. CO_2	12	28	33	37	37	42	51		

There is no big difference between the CO_2 output of the washed leaf mince and the unwashed leaf mince. It seems reasonable, therefore, to conclude that in tea fermentation the CO_2 observed arises entirely from the respiratory activity of the undamaged portions of leaf.

(2) *Freezing.* Intact cellular structure seems to be necessary for proper functioning of those enzyme systems that are usually associated with respiration [Ashford, 1934; Yudkin, 1937; Geiger, 1939]. Cellular damage markedly affects respiratory activity. As the leaf cells undergo some rupture as a result of freezing and thawing, it was thought that this method could be utilized for

effecting a separation between fermentation and respiration. Lynen [1940] has found that the respiration of animal tissues is markedly inhibited after freezing in liquid air while certain insoluble enzyme systems remain unaffected. There is an insoluble enzyme system present in tea which is largely responsible for fermentation and the effect of freezing on this enzyme system cannot be appreciable as it was found that leaf frozen in dry ice fermented normally after thawing.

In the absence of liquid air which is not available here the effect of freezing in a bath of ice and salt and also by dry ice was studied. Although the leaf became brittle on freezing, the process was rather imperfect especially in the case of withered leaf. Nevertheless freezing was found to have a significant effect on the respiration and the manometric results obtained are given in Tables 10 and 11, both the frozen and unfrozen leaf being subjected as far as possible to the same degree of mincing.

Table 10. *Effect of freezing on CO_2 output*

min. ...	10	20	30	40	50	60	70	80	90
Freezing by ice and salt:									
Frozen tissue mince, μl. CO_2	5	7	12	14	19	23	35	33	37
Unfrozen tissue mince, μl. CO_2	14	23	30	40	51	63	88	91	97
Freezing by dry ice:									
Frozen tissue mince, μl. CO_2	5	7	12	19	23	28	33	37	42
Unfrozen tissue mince, μl. CO_2	12	23	33	44	53	65	74	84	93

Table 11. *Effect of freezing on O_2 uptake for polyphenol oxidation*

min. ...	10	20	30	40	50	60	70	90
Frozen, μl. O_2	16	36	54	71	87	103	116	142
Unfrozen, μl. O_2	9	27	58	74	89	107	127	138

The degree of freezing attained, although it injured the cells, did not by itself increase the O_2 uptake, because the polyphenols in the cells of the frozen material were not exposed to atmospheric O_2. Freezing, apparently, did not supplement the degree of exposure of polyphenols attained by mechanical crushing, but there was a marked inhibition of respiration on freezing, and in one experiment the respiration of frozen whole leaf was almost negligible. The general effect, however, of freezing was to suppress the respiration without affecting the fermentation process.

Effect of glucose addition on respiration and fermentation of tea leaf

If oxidation of carbohydrate is a part of the fermentation mechanism, changes in the concentration of carbohydrate, which is the normal respiratory substrate, may be expected to have an effect on fermentation. Deb & Roberts [1940] have shown that losses of carbohydrate during the withering process in tea manufacture are unlikely to affect the rate of fermentation. Starvation for 3 days results in an appreciable fall in the respiratory activity while oxidase activity is but little affected. Thus some sort of demarcation between the two processes is already apparent and further evidence is afforded here by a study of glucose addition to the fermenting and respiring leaf.

200 mg. of leaf (either whole leaf or minced leaf) were suspended in 2 ml. glucose solution containing 10 mg. glucose. The O_2 uptakes were measured manometrically both in the case of fresh leaf and the withered leaf and typical results are given in Tables 12 and 13. Table 12 contains data for total O_2 uptake

including that required for carbohydrate oxidation while Table 13 gives figures for O_2 absorption required for polyphenol oxidation only. These latter figures are obtained on the basis of the fact that on omission of KOH, changes due to respiration are barely registered in the apparatus and therefore the results obtained represent only the uptake of O_2 due to oxidation of polyphenols. The same observations are also applicable to reactions carried out in presence of glucose, because careful manometric measurements have shown that the R.Q. during the respiration of fresh or withered leaf in presence of glucose does not change appreciably from 1·0.

Table 12. *Total O_2 uptake*

min. ...	10	20	30	40	60	80	100
Fresh whole leaf, μl.:							
Leaf + glucose	13	31	45	58	94	120	147
Leaf + water	9	22	36	45	54	85	120
Fresh leaf mince, μl.:							
Leaf + glucose	9	36	58	89	127	152	170
Leaf + water	4	27	45	80	112	127	134
Withered whole leaf, μl.:							
Leaf + glucose	13	31	40	51	80	107	138
Leaf + water	4	13	18	18	27	36	47
Withered leaf mince, μl.:							
Leaf + glucose	22	45	67	80	94	103	
Leaf + water	13	31	42	54	67	71	

Table 13. *O_2 uptake for polyphenol oxidation*

min. ...	10	20	30	40	60	90
Fresh leaf mince, μl.:						
With glucose	31	58	83	98	120	132
Without glucose	34	65	89	103	114	136
Withered leaf mince, μl.:						
With glucose	11	29	47	58	85	107
Without glucose	13	29	47	62	78	100

Addition of glucose stimulates respiration as shown by the increase in the O_2 uptake of the whole leaf. This stimulation is very marked in the case of withered leaf which may possibly be due to one or both of the two causes, namely, (1) the substrate shortage occurring during withering is made up by the addition of glucose and (2) the carbohydrate is better imbibed into the leaf as a result of an increased permeability of withered leaf [Evans, 1927].

An increase in the total O_2 uptake of the minced leaf as a result of adding glucose is indicated in Table 12. This may be due to a stimulation of either fermentation as denoted by polyphenol oxidation or of respiration. But the figures in Table 13 reveal that on omission of KOH the O_2 uptake due to fermentation remains unaffected by glucose. It must therefore be concluded that glucose, when added to minced leaf, stimulates respiration but not fermentation.

Now it has already been shown that the O_2 uptake by washed tissue mince can be completely attributed to the respiratory activity and that CO_2 output is not affected by washing the minced tissues. Stimulation of respiration must therefore be demonstrable by adding glucose to the washed tissues. Table 14

Table 14. *CO_2 production by washed leaf mince*

min. ...	10	20	30	40	60	90
With glucose, μl. CO_2	16	30	39	46	56	67
Without glucose, μl. CO_2	13	23	23	28	30	35

shows the figures for the CO_2 production with and without the addition of glucose, by 200 mg. fresh leaf mince after washing the tissues.

The increase in the respiratory CO_2 of the washed tissues on addition of glucose as shown above is of the same order as the increase in the total O_2 uptake of the unwashed tissues. It may therefore be stated that whether fermentation is taking place or not respiration is equally stimulated by glucose.

When glucose (10 mg. in 2 ml.) is added to an *in vitro* mixture of purified amorphous theotannin (2 ml. of 4·1 %) and the acetone enzyme (50 mg.) there is no increase in the O_2 uptake and also there is no CO_2 evolution. These results, which are given in Table 15, thus confirm the fact that glucose has no effect on the fermentation reaction.

Table 15. *Effect of addition of glucose to an* in vitro *enzyme-substrate mixture*

min. ...	10	20	30	60	90	120
	μl. O_2 uptake					
Reaction with glucose, KOH present	11	47	78	136	236	292
Reaction with glucose, KOH absent	13	47	74	149	232	281
Reaction without glucose, KOH present	25	40	71	147	232	285

The general conclusion from all these results can only be in support of the view that respiration and fermentation, although occurring side by side in the fermenting leaf, are really quite independent of each other.

Discussion

In the light of the present conclusions Evans's results can now be satisfactorily explained, his statement that during fermentation the normal process of respiration is not observed being strictly confined only to the damaged tissues. His observation that the volume of CO_2 produced during fermentation decreases only very slightly from the 1st hr. to the 7th shows that respiration, being a slow process, continues at almost the initial rate even long after the fermentation has come to a standstill. This obviously cannot happen if CO_2 production is due to the fermentation reaction itself. The constancy of CO_2 production and the rapid decrease in polyphenol oxidation with time together result in the fact that the ratio CO_2/O_2 approximates once again to unity, when it is only the respiratory activity that is being measured.

The independence of carbohydrate oxidation and polyphenol oxidation casts doubt on the fermentation mechanism put forward by Roberts which is already open to the criticism that most of the participating components have never been directly demonstrated in tea. The parallelism obtained by Roberts between the polyphenol oxidation and the carbohydrate oxidation was not generally found here. These appear to depend upon the nature of the leaf in the first place and then on the degree of mechanical damage. Furthermore, the inhibitory effect of the addition of ascorbic acid [Roberts, 1939] and HCN [Deb & Roberts, 1940] is different for polyphenol oxidation and carbohydrate oxidation.

The reaction in certain tissues has been explained by Szent-Györgyi & Vietorisz [1931] on the basis of the assumption that an enzyme and its substrate, an *o*-dihydroxy compound, are kept separate from one another in the uninjured cells but come into contact with each other when the cells are mechanically injured. Sutter [1936] considers that the increased O_2 uptake of the minced

tissues depends upon the facilitated diffusion of O_2. These may serve as possible alternatives to the theory of coenzyme dispersion in the damaged tissues as put forward by Roberts in his mechanism of tea fermentation. The lower carbohydrate oxidation on mincing and on dilution of the minced tea leaf tissue can be accounted for by the reduction of respiring cell surface by these operations. The extent of crushing has been shown to be a factor in explaining the disparity between the manometric results with tissue suspensions and the factory conditions.

Evidence for the occurrence of polyphenol oxidase in tea was adduced in a previous communication [Lamb & Sreerangachar, 1940], where the objections to the cytochrome and peroxidase theories were also presented. Our studies are mostly confined to fermentation and it is probable that cytochrome oxidase and peroxidase may both be functioning in the respiratory process of the tea leaf. Roberts [1940] has suggested a revision of classification of plant tissues based on the respiratory enzyme contained in the tissue and he thinks that in plants where oxidase activity is weak the respiratory enzyme may be cytochrome oxidase. This may be possible but, so far, the relation of these respiratory enzymes to tea fermentation cannot be regarded as established. It may be of interest here to mention that our acetone-treated enzyme preparation failed to show any dehydrogenase (succinic) activity. The dehydrogenase or its coenzyme may have been inactivated during preparation; yet the enzyme preparation brought about polyphenol oxidation with the greatest ease.

Reference must also be made again to our finding [Lamb & Sreerangachar, 1940] of a particular tea bush that fails to ferment. The respiratory mechanism is intact in this leaf because a plant must respire. The tannin content is also quite normal. Now, if the respiratory mechanism is indeed responsible for the fermentation of the leaf on mechanical injury, it becomes difficult to understand the failure of this leaf to ferment when minced. The requirement of an additional factor is thus clearly indicated and it has been experimentally shown that the deficient factor is indeed the polyphenol oxidase which is directly responsible for fermentation.

Summary

The CO_2 produced by the fermenting leaf of the tea plant is shown to arise from the respiratory activity of the undamaged portions of leaf. *In vitro* enzymic oxidation of tea polyphenols is not accompanied by any CO_2 production in the presence of carbohydrates. Respiration and tea fermentation are thus found to be two separate processes occurring side by side in the damaged tea leaf but quite independent of each other.

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126. A SIMPLE COLORIMETRIC METHOD FOR THE ESTIMATION OF HAEMOLYSIS AND ITS APPLICATION TO THE STUDY OF STREPTOLYSIN

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THE method to be described arose as the result of an investigation[2] of a haemolysin produced by group A haemolytic streptococci ('streptolysin O'). In the course of this work, many measurements of the haemolytic activities of different streptolysin O preparations had to be made, and the need was felt of a method for measuring haemolysis more accurate than those in common use.

The method of 'serial dilutions' is usually adopted for the measurement of haemolytic activity. Falling amounts of haemolysin solution are incubated in a series of tubes with constant amounts of a red blood cell suspension; the 'end-point' is then taken as the last tube in which complete haemolysis occurs, as judged by eye. Widely adopted as this method is, it is extremely inaccurate. If, as is usual, the serial dilutions are so spaced that each tube contains half as much haemolysin as the preceding one, then the possible error is anything up to 100%. This error cannot be greatly reduced by a closer spacing of the dilutions, for this makes it more difficult to determine the end-point by visual inspection. In addition to this there are other sources of error in the method as it is usually practised, such as inadequate control of *p*H and other factors to be discussed below.

Other more refined methods have occasionally been used for measurement of haemolytic activity; these have mostly been too time-consuming or have needed too complicated apparatus to come into routine use among pathologists.

Eagle [1929], studying the haemolysis of sensitized red cells by complement, observed the time taken for complete haemolysis of a certain amount of red cells, which was a function of the amount of complement present. The author is inclined to doubt the accuracy of this method unless some form of optical apparatus is used to assist in determining the exact moment of complete haemolysis, which is rather difficult to determine by the naked eye alone.

A more refined application of the same principle has been designed by Ponder [1932] and applied to the study of haemolysis by saponins etc. In this method the red cells are mixed with haemolysin in one cell of a Stufenphotometer immersed in a constant temperature bath and with an automatic stirrer to prevent the red cells settling. The amount of light passing through the suspension is determined from moment to moment; this allows the time taken for complete haemolysis and for any degree of partial haemolysis to be measured with high accuracy. This is probably the most sensitive arrangement for the measurement of haemolysis yet designed, but it has the disadvantage of needing rather complicated apparatus.

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[2] Reported in the following paper by Herbert & Todd [1941].

Vles *et al.* [1931] incubated red cells with amounts of haemolysin sufficient to cause only partial haemolysis in a given time; after centrifuging unlysed red cells the degree of haemolysis was measured by determining the amount of haemoglobin in the supernatant. This was done by measuring the extinction coefficient of the absorption band at 542 mμ in a visual spectrophotometer. This method is accurate but again needs complicated apparatus and in addition is very time-consuming.

It may be pointed out that the method of Vles *et al.* [1931] and the customary method of 'serial dilutions' both measure the amount of haemolysis occurring in a given time, while the method of Eagle measures the time taken to effect a certain amount of haemolysis; the apparatus of Ponder may be used in either way.

The method to be described is based on the first principle, and is similar to that of Vles *et al.* in that the extent of partial haemolysis in a given time is measured by determination of the amount of haemoglobin set free from the haemolysed red cells; this is done however by a colorimetric method to be described below, which is extremely rapid and simple and does not need complicated apparatus.

Experimental

Briefly, the method consists in measuring out into a series of tubes falling amounts of the haemolysin solution whose activity is to be measured, and incubating them all with a standard amount of a red cell suspension; after incubation the tubes are centrifuged and the amounts of haemoglobin in the supernatant measured. Other important features of the method are as follows.

(1) *Control of* p*H*. The medium in which haemolytic tests are usually carried out is 'normal' saline; in other words it is a substantially unbuffered medium. This is liable to lead to error since the haemolytic activities of most haemolysins vary with the *p*H, and the *p*H at which they exert their maximum activity is not necessarily at neutrality. The haemolytic activity of streptolysin O, for example, is maximum at *p*H 6·5 and falls off quite sharply on either side of this *p*H [Herbert & Todd, 1941].

For accurate work, therefore, haemolysis tests should be carried out in isotonic buffer solutions. For measurements on streptolysin O a phosphate buffer of *p*H 6·5 (68 ml. 0·158 *M* Na_2HPO_4 + 32 ml. 0·158 *M* NaH_2PO_4) was used; this buffer, which is itself isotonic, was mixed with an equal volume of 0·9 % NaCl. This isotonic phosphate-NaCl mixture was used for washing and suspending the red cells, and also for diluting haemolysin solutions. For other haemolysins, of course, buffers of different *p*H and hence of different molarity might have to be used. As far as I am aware, the literature contains no references to isotonic phosphate buffers; for the purposes of this work, the data of Grollmann [1931] on the vapour pressures of *M*/15 phosphate buffer mixtures were used to calculate the molarities of buffers isotonic with 0·9 % NaCl.

(2) *The use of red cell suspensions of an exactly standardized strength.* Red cell suspensions were standardized by colorimetric estimation of their haemoglobin content, and the standard suspensions used always contained exactly 7·5 mg. haemoglobin per ml. This figure was chosen because it corresponds very closely (for rabbit's red cells) with the 2½ % by volume suspension commonly used by pathologists. Standardization by measuring the haemoglobin content, however, is much more accurate than by measuring the volume of the red cells.

The standard conditions used throughout this work for measuring haemolytic activity were as follows.

Varying amounts of the haemolysin solution, made up to 1 ml. with isotonic saline-phosphate *p*H 6·5, were mixed with 1 ml. of a standard red cell suspension and incubated for 30 min. at 38°. The tubes were then immediately centrifuged and the amounts of haemoglobin in the supernatant fluids determined colorimetrically. When adding the red cell suspension to the haemolysin it is important (in the case of streptolysin O at least) that mixing should be immediate and complete; the red cell suspension is therefore rapidly blown into the haemolysin solution from a 1 ml. 'blow-out' pipette.

The amount of haemolysin that under these conditions liberates 3·75 mg. haemoglobin (corresponding to haemolysis of 50 % of the standard dose of red cells) is called one haemolytic unit (H.U.). Thus if 0·001 ml. of a certain haemolysin solution caused exactly 50 % haemolysis under the standard conditions, then the solution would contain 1000 H.U./ml.

The haemoglobin estimations were carried out in a Leitz colorimeter. The principle of the method is that if a suitable green filter is used in the eyepiece of the colorimeter, a haemoglobin solution may be matched against a neutral grey filter of known density, which serves as a permanent standard and obviates the necessity for continually preparing fresh standard haemoglobin solutions. The no. 7 green filter in the eyepiece of the Leitz colorimeter was used, together with Ilford neutral grey filters of densities 1·0 and 1·5. With the above green filter haemoglobin solutions could be matched most perfectly against the neutral grey filters, and the matching was found to be very sharp and much easier than matching two haemoglobin solutions in an ordinary colorimeter without a filter in the eyepiece.

Micro (1 ml.) cups were used in the colorimeter, and coupled plungers, which move together so that the two liquid columns are always the same height. The left-hand cup contains water, and the right-hand cup the solution whose haemoglobin content is to be measured; a neutral grey filter is interposed between the left-hand cup and the eyepiece. This arrangement is not strictly necessary, but is useful especially if the haemolysin solution contains some coloured substance other than haemoglobin; in such a case the coloured solution can be added to the left-hand cup instead of water, when its effect is automatically compensated for, since the two liquid columns are always the same height.

The Ilford neutral grey filters have densities guaranteed by the makers to be accurate to within 1 %; however, they cannot be used directly to measure the strength of haemoglobin solutions since the light transmitted by the eyepiece filter is not perfectly monochromatic, but stretches over a comparatively wide region of the spectrum. The neutral grey filters must therefore first be calibrated in terms of haemoglobin. This was done by matching them in the colorimeter in the manner described against a solution of haemoglobin of known strength, determined by measuring the extinction coefficient of the 575 mμ band in a Hilger visual spectrophotometer. This has only to be done once; the neutral grey filters then constitute permanent standards, and the strength of unknown haemoglobin solutions matched against them can be read off directly in terms of mg. haemoglobin per ml.

A rheostat is incorporated in the Leitz colorimeter by which the intensity of the light may be varied; by the use of this and of a series of neutral grey filters of different densities, a wide range of haemoglobin concentrations can be measured.

It will be apparent that the method described above is a form of absolute colorimetry, and that any other type of absolute colorimeter such as a Stufen-photometer or a photoelectric colorimeter could be substituted for the arrangement described. Alternatively a simple colorimeter, preferably with a green

filter in the eyepiece, could be used, and the unknown haemoglobin matched against a standard haemoglobin solution of known strength, as in ordinary colorimetry. The disadvantage of this method is the constant need to prepare fresh haemoglobin solutions.

The above method was also used to prepare the standard red cell suspensions. 1 ml. of a concentrated (*ca.* 20 %) suspension of red cells, previously washed three times in isotonic buffer, was laked in 25 ml. of distilled water, and after centrifuging down stromata the haemoglobin content was measured as above. The concentrated suspension was then diluted until it contained exactly 7·5 mg. haemoglobin per ml. Rabbit red cells were used throughout. The red cells were kept in the refrigerator and were not used after they were 3 days old.

Some examples will now be given of the application of the method to the measurement of the haemolytic activity of streptolysin O preparations. It should be mentioned that this haemolysin is unusual in that it is only active in the presence of reducing agents such as sodium hydrosulphite, cysteine, thiolacetic acid etc., and is inactivated by oxidation. This phenomenon is described in detail by Herbert & Todd [1941]; since the present paper is chiefly concerned with the method of estimation of haemolysis, it will suffice to say here that all the experiments to be described were carried out on fully activated streptolysin.

Fig. 1 shows an experiment in which varying amounts of a streptolysin O solution, activated with thiolacetic acid, were incubated with red cells, and the degree of haemolysis measured in each case. The upper dotted line, at 7·5 mg. haemoglobin, indicates complete haemolysis; the lower, at 3·75 mg. haemoglobin indicates 50 % haemolysis. It will be seen from the curve how the amount of streptolysin which liberates exactly 3·75 mg. haemoglobin in 30 min. at 38° can be found, and hence the number of H.U./ml.

Fig. 2 shows similar experiments with four different haemolysin solutions of different strengths. In Fig. 3 the results of Fig. 2 are replotted, the scales of the abscissae being adjusted so that the mid-points of all four curves coincide; it will be seen that when this is done all the curves coincide completely throughout their length.

Many hundreds of such determinations have been carried out in the course of this work, and in all cases, curves of the same characteristic sigmoid shape have been obtained.

The nature of the reaction underlying the peculiar shape of this curve can be seen from Fig. 4, in which the experimental data of Fig. 1 are plotted in a different way. Instead of the degree of haemolysis ('H') being plotted against the concentration of streptolysin ('L') as in Fig. 1, in Fig. 4 $d\mathrm{H}/d\mathrm{L}$ is plotted against L. The curve then appears as what it really is—a frequency diagram of the red cell population, expressing the resistance of different red cells to haemolysis by different concentrations of streptolysin. It is to be presumed that for each individual red cell there is a critical concentration of streptolysin below which it is not haemolysed and above which it is completely haemolysed in a given time. This critical concentration will of course be different for different red cells, varying with their age, size, thickness of cell membrane etc.

Haemolysis of a single red cell, it must be presumed, is an 'all-or-none' process; degrees of partial haemolysis are only possible with a heterogeneous collection of many red cells. On this view, if the action of streptolysin O could be tested on red cell suspensions in which all the red cells were exactly alike, the resulting curves instead of being sigmoid in shape like the curves of Figs. 1–3, would be a series of vertical straight lines.

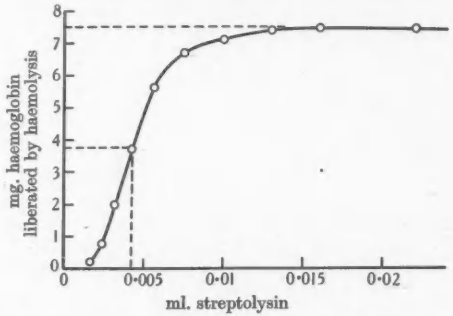

Fig. 1. Concentration-haemolysis curve for streptolysin O. In this and all succeeding curves, experimental conditions were as follows: the amount of streptolysin solution shown was incubated for 30 min. at 38° with 1 ml. of a red cell suspension containing 7·5 mg. haemoglobin per ml., and the amounts of haemoglobin liberated by haemolysis measured as described in the text.

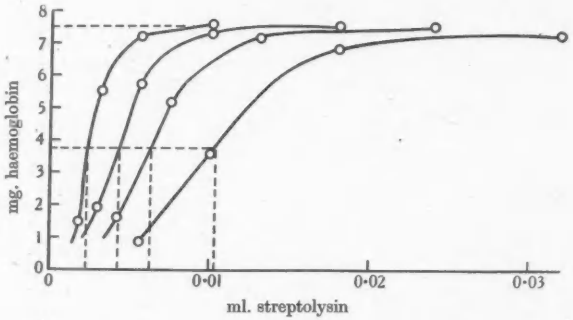

Fig. 2. Concentration-haemolysis curves for streptolysin O. The four curves represent four different streptolysin preparations.

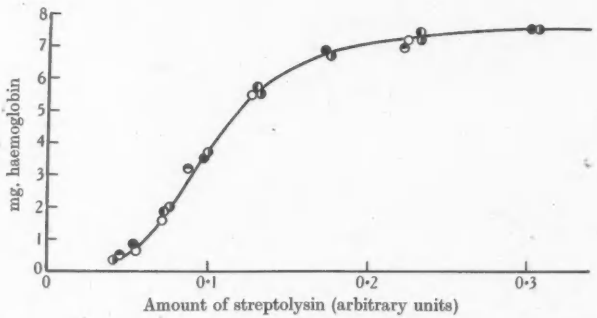

Fig. 3. For explanation see text. ○●◑◐◓ represent the four curves of Fig. 2 and Fig. 1.

A study of Figs. 1–3 reveals another point of more immediate practical importance. The slope of these curves is greatest in the region of about 50% haemolysis, while in the region of almost complete haemolysis the curve flattens out and becomes nearly asymptotic.

In this region of the curve, large variations in the amount of haemolysin added produce but small changes in the degree of haemolysis; e.g. a 50% increase in the amount of haemolysin added only increases the degree of haemolysis from 96 to 100%; in the middle region of the curve, however, a 6% increase in the amount of haemolysin added will increase the degree of haemolysis from 48 to 52%.

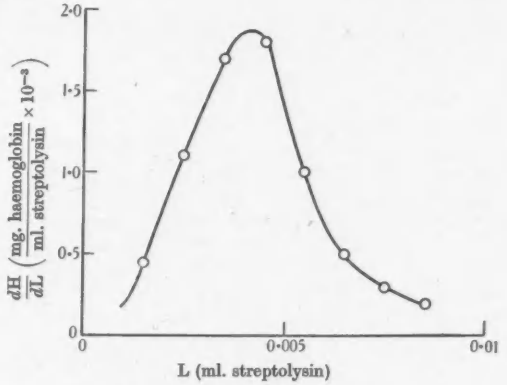

Fig. 4. For explanation see text.

This clearly shows how inaccurate it is to measure the activity of haemolysins by taking complete haemolysis as an end-point; it is far more accurate to take 50% haemolysis as an end-point, even if the degree of haemolysis is estimated by visual inspection only.

In this work, the activity of an unknown streptolysin solution was determined by setting up (usually) 5 tubes containing falling amounts of haemolysin at 25% intervals, and after incubating with red cells etc. as described above, constructing a curve similar to Fig. 1; the amount of haemolysin necessary to cause exactly 50% haemolysis of the standard dose of cells can then be determined by interpolation. Alternatively this can be done by reference to the standard curve of Fig. 3.

Theoretically the activity of an unknown streptolysin solution could be determined by putting up a single tube and referring to the standard curve; in practice it was considered more accurate to put up a series of tubes and work by interpolation.

The above method has been in routine use for a year and has proved extremely satisfactory. It is about ten times more accurate than the method of serial dilutions commonly employed, the mean error being about $\pm 5\%$.

The method contains a possible source of error which is worthy of mention, namely, that haemolytic activity is being measured by a biological reagent, red blood cells, whose natural variation might be a disturbing factor. Prigge [1937], discussing the haemolysins of *Cl. welchii*, lays great stress on this point, and states that the same haemolysin solution may have apparently different activities

if measured with different red cell suspensions, or with the same red cell suspension at different times. He therefore recommends that whenever an unknown haemolysin is to be tested, a parallel experiment should be put up with a 'standard' haemolysin preparation, and the activity of the unknown expressed in terms of the standard. Theoretically this is a sound principle; in practice I have not found it necessary to take this precaution, since I have never observed such variation in different batches of red cells as Prigge apparently found.

Table 1 shows some measurements made on the same streptolysin solution with the same batch of red cells on the same day. In all cases the agreement

Table 1. *Haemolytic activities of streptolysin O preparations tested repeatedly on the same red cells*

Streptolysin batch no.	Haemolytic activity found (H.U./ml.)
(3)	278, 278, 280, 283; mean 279·8
(6)	1210, 1200, 1190; mean 1202
(9)	1520, 1510; mean 1515
(10)	800, 800, 790; mean 796·7

Table 2. *Haemolytic activities of various streptolysin O solutions tested upon different occasions*

Date tested	Haemolytic activity found (H.U./ml.)
I. Purified streptolysin preparation 8	
12. xii. 40	1870
13. xii. 40	1850
13. xii. 40	1840
15. xii. 40	1760
15. xii. 40	1940
15. xii. 40	1745
II. Purified streptolysin preparation 9. Stored in refrigerator at 2°	
13. ii. 41	890
14. ii. 41	910
19. ii. 41	877
22. ii. 41	870
23. ii. 41	890
1. iv. 41	666
1. iv. 41	625
2. iv. 41	588
2. iv. 41	575
3. iv. 41	564
6. iv. 41	567
16. iv. 41	524
III. Purified streptolysin preparation 13. Kept frozen solid at −10°	
10. vi. 41	877
11. vi. 41	862
3. ix. 41	862
4. ix. 41	877
20. x. 41	852

between duplicates is very good. Table 2 shows experiments in which streptolysin solutions have been repeatedly tested on different occasions with different batches of red cells. Here again the agreement is good. In the measurements on preparation 9 in Table 2 it is interesting to compare the series made in February, 1941, which all give about the same value, with those made two months later; the solution had stood in the refrigerator at 2° in the interval and its activity had decreased, but the second series of measurements, though all lower than

the first, are in good agreement with each other. Preparation 13, in the same Table, had been kept frozen solid at $-10°$, and in this case a series of measurements made over an interval of four months (and moreover, in this case not only with different samples of blood, but with blood from different animals) all give values in close agreement.

It is difficult to reconcile these results with those of Prigge; one can only conclude either that there is much more variability in the red cells of sheep, which he used, than in those of rabbits, which I used, or else that some other source of error was present in his technique.

Although I have not found it necessary to allow for biological variation by running each measurement of an unknown haemolysin solution in parallel with a measurement on a standard, it is obvious that if necessary this could easily be done, and the principle of the method is not affected thereby. In this case unknown and standard solutions would both be measured in the manner above described, on the same red cells at the same time. If the amounts of unknown and standard haemolysin solutions required to cause haemolysis of exactly 50 % of the standard dose of red cells were x ml. and y ml. respectively, and if the standard had S units per ml., then the unknown would have $S.y/x$ units per ml.

It is hoped that the method of determining haemolytic activity described in this paper will be found useful by pathologists. It has been used routinely in this laboratory over the last twelve months and has been applied to the study of streptolysin O, streptolysin S, and the haemolysins of *Cl. welchii* and *Cl. tetani*. It should be applicable to the study of all bacterial haemolysins, and also of haemolysis by complement and by saponin etc.

SUMMARY

A simple and accurate method for the measurement of haemolysis is described, together with some results obtained by the application of the method to the study of streptolysin O.

The author is indebted to the Medical Research Council for a research grant, and to Drs E. W. Todd and W. E. van Heyningen for much helpful discussion and advice.

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127. PURIFICATION AND PROPERTIES OF A HAEMOLYSIN PRODUCED BY GROUP A HAEMOLYTIC STREPTOCOCCI (STREPTOLYSIN O)

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IT has long been known that many strains of haemolytic streptococci produce filterable haemolysins. Work on this subject has been complicated by the facts that (1) either or both of two different haemolysins may be produced according to the conditions of growth and the bacterial strain, (2) one of these haemolysins is only active in the presence of certain reducing agents such as sodium hydrosulphite, cysteine and other —SH compounds.

Neill & Mallory [1926] first made the important observation that the haemolytic activity of fresh broth filtrates in which haemolytic streptococci have grown, rapidly disappeared on standing in air, though less rapidly in the absence of oxygen. If reducing agents such as hydrosulphite were then added, the haemolytic activity was restored to the original level or even higher.

These observations were confirmed and extended by Todd [1932] who has called this haemolysin 'streptolysin O' [Todd, 1938] to indicate susceptibility to oxidation and reduction. Todd [1939] has also shown that streptolysin O is produced by most strains of group A streptococci when grown in a serum-free broth such as the glucose-bicarbonate-phosphate broth of Todd & Hewitt [1932]; it is also produced by group C strains from human infections and by group G strains, but not by streptococci of other groups. It is antigenic, and potent antisera may be made by injecting such broth filtrates into horses or rabbits. The streptolysins produced by all types and strains of group A streptococci are serologically identical, and the antibody to any one of them will neutralize all of them to the same extent.

If haemolytic streptococci are grown in media containing serum a second haemolysin is also produced, whose activity is not affected by oxidation and reduction. Todd [1938] named this 'streptolysin S' to indicate that it is only produced in the presence of serum, and has shown that streptolysins O and S are two entirely distinct haemolysins and are neutralized by different antibodies.

So far these haemolysins have been studied mainly from a serological standpoint, and little chemical work has been carried out, apart from a single paper by Smythe & Harris [1940]. These authors were able to purify streptolysin O to a considerable extent but the details of procedure, yields etc., given in their paper are so scanty that it is impossible to repeat their methods. Their purified products were non-dialysable and appeared to be protein in nature, though they also contained *ca.* 60 % carbohydrate.

The object of the present work was to isolate streptolysin O in as pure a state as possible with a view to making a detailed study of its chemical properties.

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Besides the two streptolysins already mentioned, haemolytic streptococci produce a number of other toxins, e.g. the scarlet fever toxin, a leucocidin, a 'diffusing factor' etc., and it is at present difficult to say whether these various toxins are the same or different, and to what extent any of them are associated with virulence. This is a problem which can only be settled by isolation of the different toxins in a pure state.

Experimental

(1) *Methods*

(*a*) *Determination of haemolytic activity.* The haemolytic activity of streptolysin O solutions was determined by the accurate colorimetric method described in the preceding paper [Herbert, 1941]. The standard conditions therein described were used throughout this work except where otherwise stated, and the same haemolytic unit (H.U.) is meant in both papers. (It should be pointed out that this is different from the M.H.D. used by Todd in previous work on streptolysin O. A solution containing 100 Todd M.H.D./ml. has about 250 H.U./ml.)

As previously mentioned, streptolysin O has almost no haemolytic activity except in the presence of certain reducing agents, and these must always be added before testing. Thiolacetic acid was used routinely. 0·1 ml. of B.D.H. 90% thiolacetic acid is added to *ca.* 2 ml. of distilled water, neutralized to *p*H 6·5 with *N* NaOH and diluted to 5 ml.; this gives an approx. *M*/5 solution. 0·5 ml. of this was added to 2 ml. of the streptolysin solution to be tested; after 10 min. at room temperature it was diluted with isotonic phosphate-NaCl; appropriate amounts were measured out, and incubated with red cells in the usual way.

Neutralized *M*/5 solutions of cysteine or Na_2SO_3 were occasionally used instead. Any of the above reagents is more convenient than sodium hydrosulphite which has been commonly used up to now. The great speed with which the latter is oxidized in air, and the fact that its solutions become extremely acid on oxidation, renders it less convenient than the other reagents mentioned.

(*b*) *Dry weights.* Throughout this work, the purity of any streptolysin O preparation is expressed in terms of H.U./mg. dry weight. Dry weights were determined by dialysing a known amount of solution until salt-free, measuring an aliquot into a tared glass dish, freezing at $-10°$, drying from the frozen state *in vacuo* over H_2SO_4 and reweighing.

(*c*) *Chemical methods.* Total carbohydrate in protein preparations was determined by the orcin method of Pirie [1936]. Phosphorus was determined by the method of Fiske and Subbarow as modified by Horecker *et al.* [1940]. Arginine was determined according to Weber [1930]. Sulphur was determined by the method of Denis & Reed [1926] as modified by Dr E. M. Crook (private communication).

(2) *Method of purification of streptolysin O*

The starting material was filtrates of cultures of group A streptococci grown in the broth of Todd & Hewitt [1932].

This broth was found to contain *ca.* 30 g./l. of total solids and about 10 g./l. of non-dialysable material, almost all of which is precipitated by full saturation with $(NH_4)_2SO_4$. These figures are not greatly changed after the organism has grown in the broth for 16 hr. From the point of view of purification, this high protein content constitutes a disadvantage which is, however, outweighed by the fact that this broth is the best so far evolved for the production of streptolysin O.

Except where otherwise stated the well-known 'Richards' strain (group A, type 3) was used. Stock cultures were preserved in dried form and inoculated

into broth tubes which were incubated 6 hr.; 2 l. batches of broth in Winchester bottles were inoculated with *ca.* 2 ml. of the 6 hr. culture, and incubated 16 hr. at 37°, tested for contamination and, if uncontaminated, filtered through Pasteur-Chamberland F candles.

The method of purification adopted involves fractionation with $(NH_4)_2SO_4$, adsorption and elution from calcium phosphate gel, followed by adsorption and elution from alumina Cγ. Full details of a typical preparation are given below.

The starting material was 4190 ml. of broth, containing 167 H.U./ml., or 700,000 H.U. in all.

Stage 1. 1510 g. solid $(NH_4)_2SO_4$ were added, making the solution 0·57 saturated. The bulky, flocculent precipitate was filtered on large Büchner funnels and sucked as dry as possible; the filtrate was discarded. The precipitate was dissolved in 400 ml. of *M*/15 phosphate *p*H 7·2, giving 480 ml. of a rather brown, sticky solution, containing 1110 H.U./ml., or 534,000 H.U. in all.

Stage 2. To the above solution, which was 0·14 saturated with $(NH_4)_2SO_4$, were added 212 ml. of saturated $(NH_4)_2SO_4$, slowly and with good stirring: final concentration 0·37 saturated. The dark brown, very sticky precipitate was centrifuged off and discarded, and the water-clear, only slightly coloured, solution dialysed overnight against running water: final volume 915 ml., containing 356 H.U./ml., or 326,000 H.U. in all.

Stage 3. To the above solution were added 30 ml. *M*/2 phosphate buffer *p*H 7·2, followed by 12·5 ml. *M* calcium acetate. After 20 min. the calcium phosphate precipitate was centrifuged, and the supernatant, which contained only 3 H.U./ml., discarded. The streptolysin was all adsorbed on the calcium precipitate, and was eluted therefrom with three successive 25 ml. portions of 0·25 saturated $(NH_4)_2SO_4$. The combined eluates were dialysed against distilled water: final volume 112 ml. containing 2560 H.U./ml., or 287,000 H.U. in all.

Stage 4. Adsorption and elution from calcium phosphate was now repeated. 10 ml. *M*/2 phosphate *p*H 7·2 were added to the above solution followed by 4 ml. *M* calcium acetate. (The *p*H if necessary was then adjusted to 6·5 with dilute acetic acid.) After 20 min. the calcium phosphate precipitate was centrifuged and eluted 5 times with successive portions of 0·25 saturated $(NH_4)_2SO_4$; the combined eluates were dialysed: final volume 31·5 ml., containing 6000 H.U./ml., or 189,000 H.U. in all.

Stage 5. To the above solution were added 5 ml. of an alumina Cγ gel containing 14·2 mg. Al_2O_3/ml., and the *p*H adjusted to 6·5 with dilute acetic acid. The alumina was centrifuged down, the supernatant discarded and the alumina was eluted twice with 4 ml. portions of ammoniacal $(NH_4)_2SO_4$ (97·5 ml. saturated $(NH_4)_2SO_4$, 2·5 ml. ammonia sp. gr. 0·880, diluted to 400 ml.) and the combined eluates were exhaustively dialysed: final volume 10 ml., containing 12,100 H.U./ml., or 121,000 H.U. in all.

The yields and relative purity of the streptolysin at the different stages of purification are shown in Table 1.

The starting material contained altogether 700,000 H.U. the relative purity of which, on a dry weight basis was 18·4 H.U./mg. The final product, 121,000 H.U. in all, had a relative purity of 3050 H.U./mg. dry weight. The degree of purification was therefore 166-fold, with an overall yield of streptolysin O of 17·3 %.

The final product is undoubtedly not completely pure; however, the small quantity of material available (39·7 mg. of purified streptolysin from 4 l. of broth) made further purification impracticable.

Table 1. *Purification of streptolysin O*

Stage	Volume ml.	H.U./ml.	Total H.U.	Dry weight mg./ml.	Total dry weight	H.U./mg.
Broth	4190	167	700,000	9·08	38·1 g.	18·4
1	480	1,110	534,000	34·2	16·4 g.	33·2
2	915	356	326,000	7·06	6·45 g.	50·5
3	112	2,560	287,000	3·5	392 mg.	732
4	31·5	6,000	189,000	3·1	97·6 mg.	1935
5	10	12,100	121,000	3·97	39·7 mg.	3050

(3) *Properties of the purified streptolysin O*

(*a*) *Analysis.* The final product from the preparation described above, which had been dialysed against many changes of glass-distilled water, was dried from the frozen state *in vacuo* over H_2SO_4. (Found: C, 46·7; H, 6·8; N, 16·8; S, 2·34; P, 0·053; ash, 3·6; total carbohydrate, 2·6 %.) The C, H, and N figures are micro-analyses by Dr Weiler, Oxford. The frozen-dried substance lost a further 6·35 % of H_2O on drying to constant weight *in vacuo* at 100°; the above figures are corrected for this.

Other preparations gave closely similar results. The ash content was usually lower than that of this particular preparation, e.g. 1·0–1·5 %. The above are typical analytical figures of a protein, and all evidence favours the view that streptolysin O is in fact a protein. Purified preparations gave all the usual colour reactions for proteins, e.g. the biuret reaction and tests for tyrosine and tryptophan. They also gave a strong Sakaguchi reaction, and quantitative analysis by the Weber method showed the above preparation to contain 4·7 % of arginine.

The protein gave a negative nitroprusside reaction before, and a positive reaction after, treatment with KCN; i.e. it presumably contains —S—S— linkages which can be reduced to —SH groups.

The preparation described above was from a 'Richards' strain. In one case, broth from a culture of strain 0·89 Matt Attenuated was put through the first four stages of the purification process described above. It behaved similarly in all respects, and a highly purified product was obtained, which gave similar analytical figures to the above and behaved in the same way. While the matter has not been closely investigated, it seems probable that the streptolysins O produced by all types of group A streptococci are very closely similar, if not identical, chemically as well as immunologically.

(*b*) *General properties.* Purified streptolysin O preparations on drying from the frozen state form a white fluffy powder. It is readily soluble in distilled water or dilute salt solutions; the exact solubility is unknown but 5 % solutions can easily be obtained. Such solutions are crystal clear and usually completely colourless, though sometimes faintly yellow. It is precipitated by trichloroacetic acid and the usual protein precipitants, by high concentrations of $(NH_4)_2SO_4$, and by alcohol and acetone. It is coagulated on boiling.

Fairly concentrated solutions are perfectly stable for weeks at 0°, though they lose an appreciable part of their activity in two months. Very weak solutions (0·001 mg./ml.) are less stable, and may be irreversibly inactivated in a few hours at room temperature or even less at 37°, presumably owing to surface denaturation, since the purified material is much less stable than crude streptolysin-broth in this respect in weak solutions, though equally stable in strong solutions. At all stages of purity, boiling for 2 min. causes complete and irreversible inactivation; so also does treatment with strong acids and alkalis. Frozen-dried preparations appear to retain their activity indefinitely, and the

drying process itself causes no loss of activity. Some data on relative stabilities of purified preparations under different conditions may be found also in Table 2 of the preceding paper.

(c) *Activation of streptolysin O by reducing agents.* As already stated, the haemolytic activity of streptolysin broth filtrates was extremely small if tested in the absence of cysteine or some other reducing agent. The haemolytic activity after activation with cysteine was usually 20–40 times that of the unactivated streptolysin broth, e.g.

	Haemolytic activity (H.U./ml.)	
Broth batch no.	Tested without activator	With *M*/25 cysteine
64	3	125
68	4	149
69	3	84

The same also applies to purified streptolysin O at all stages of purification.

The activating effect of a large number of substances was tested with results shown in Table 2. In each series of experiments, one with cysteine was included; the relative degrees of activation brought about by the various substances tested are then expressed as percentages of the activity with cysteine, which is given an arbitrary value of 100 %.

Table 2. *Activation of streptolysin O by various reducing agents*

In all cases, 2 ml. of a streptolysin O solution, containing *ca.* 1 mg. of purified haemolysin, was added to 0·5 ml. of a neutralized *M*/5 solution* of the reducing or oxidizing agent to be tested. After 10 min. at room temperature, the mixture was diluted with saline-phosphate *p*H 6·5 to the appropriate strength, and its haemolytic activity measured in the usual way.

Controls were done on all the substances tested, by adding 1 ml. of a *M*/25 solution to 1 ml. red cells; in no case was any haemolysis observed.

The three experiments were made with different streptolysin preparations.

Oxidizing or reducing agent	Haemolytic activity H.U./ml.	Relative activity (cysteine = 100)
	Exp. 1	
Cysteine	1850	100
Thiolacetic acid	1840	99·5
β-Thiolpropionic acid	1760	95·2
Reduced glutathione	1745	94·5
Na_2SO_3	1940	104
None	97	5·2
	Exp. 2	
Cysteine	890	100
o-Thiolbenzoic acid	934	105
H_2S	800–1000†	90–110†
$Na_2S_2O_4$	890	100
KCN	132	15
$Na_2S_2O_3$	77	8·6
$K_4Fe(CN)_6$	33	3·7
Ascorbic acid	32	3·6
None	32	3·6
	Exp. 3	
Cysteine	856	100
Cystine	6	0·7
Oxidized glutathione	3	0·35
Dithiodiacetic acid	8	0·94
H_2O_2	5	0·58
None	18·6	2·2

* In the case of H_2S, a saturated solution was used; in the case of cystine a *M*/5 suspension.

† When H_2S is used, the formation of sulphaemoglobin and deposition of colloidal S make accurate colorimetric readings impossible; these figures are approximate.

The substances tested can be divided into three groups so far as their activating effects are concerned:

(1) All the —SH compounds tested[1] (cysteine, reduced glutathione etc.), and also H_2S, $Na_2S_2O_4$ and Na_2SO_3 have the same maximum activating effect (within the limits of error of the methods used).

(2) The —S—S— compounds corresponding to the above —SH compounds (cystine, oxidized glutathione, dithiodiacetic acid etc.) and also ascorbic acid and ferrocyanide have no activating effect at all; the former group of compounds in fact decrease the small blank activity.

(3) KCN and $Na_2S_2O_3$ occupy an intermediate position. They bring about a definite activation, quite outside the limits of experimental error (haemolytic activity being increased 2–5 times above the control), but much smaller than that brought about by substances of group (1) which is *ca.* 30 times greater than that of the control.

The activation of streptolysin O by cysteine etc. is a very rapid process at room temperature; e.g. if 0·5 ml. *M*/5 cysteine is added to 2 ml. streptolysin and then diluted and tested immediately, the haemolytic activity found is the same as when the streptolysin is kept 30 min. with the cysteine before testing.

No detailed study was made of the amount of cysteine (or other reducing agent) required for the activation of a given amount of streptolysin. The method adopted in practice was to keep 2 ml. of streptolysin solution containing *ca.* 200–2000 H.U. for a short time with 0·5 ml. of a *M*/5 solution of the reducing agent; the mixture was then diluted until it contained *ca.* 1 H.U./ml. The final concentration of reducing agent is therefore very small; however, if the dilution is made instead with saline-phosphate containing *M*/25 cysteine, thiolacetate or Na_2SO_3, exactly the same results are obtained.

The activation produced by reducing agents is completely reversible; this can be shown in the following way. If cysteine is added to a streptolysin solution, the initially low haemolytic activity is of course greatly increased. If the streptolysin-cysteine solution is now dialysed until the cysteine is all removed, the haemolytic activity falls again to its original low level; but if cysteine is once more added, the haemolytic activity is again increased as much as before. The following figures illustrate such an experiment:

	Haemolytic activity H.U./ml.
(1) Unactivated streptolysin O solution	6·7
(2) The same after adding cysteine to a final concentration of *M*/25	422
(3) Streptolysin-cysteine solution after 24 hr. dialysis (nitroprusside test negative)	9·0
(4) Dialysed solution after adding cysteine (*M*/25)	400

(N.B. the values in (3) and (4) are corrected for the volume increase occurring on dialysis.) Another portion of the streptolysin-cysteine solution, instead of dialysing, was kept for 24 hr. at room temperature; the haemolytic activity had not decreased. Some of the cysteine had been oxidized to cystine, but there was still sufficient to give a most intense nitroprusside reaction.

[1] It is interesting that the aromatic —SH compound *o*-thiolbenzoic acid, in which the —SH group is directly attached to the benzene ring, has exactly the same activating effect as the aliphatic —SH compounds tested.

The most obvious hypothesis to explain all the above facts is that streptolysin O is a protein containing —S—S— linkages, which can be reversibly reduced to —SH groups. The —S—S— form of the protein may be assumed to be haemolytically inactive while the —SH form is active. The activation of inactive streptolysin by cystine, reduced glutathione, Na_2SO_3 etc., is then to be interpreted as a reduction of the protein —S—S— groups to —SH by the above compounds:

$$\underset{\text{(inactive)}}{\text{Streptolysin}\begin{matrix}\diagup \text{S} \\ | \\ \diagdown \text{S}\end{matrix}} + 2R\text{SH} \rightleftharpoons \underset{\text{(active)}}{\text{Streptolysin}\begin{matrix}\diagup \text{SH} \\ \\ \diagdown \text{SH}\end{matrix}} + R\text{SS}R$$

The —SH groups of the inactive haemolysin must be assumed to be, like most —SH groups, autoxidizable in air. This oxidation cannot take place when large amounts of cysteine (or other reducing agent) are present, since the cysteine —SH groups, so long as they are present in excess, will keep the sulphur linkages of the protein in the reduced state by mass action effect, and will be oxidized instead. When the cysteine is removed, by dialysis or any other means, the protein —SH groups are slowly oxidized by atmospheric oxygen.

The unactivated preparations of streptolysin always possessed a small haemolytic activity. This, however, was relatively diminished in the course of purification; thus in the preparation described in § 2, the following figures were found:

	Haemolytic activity (H.U./ml.)		
	With thiolacetic acid	No activator	Ratio
Streptolysin broth	167	4·2	40/1
Purified streptolysin, stage 3	2,560	33	78/1
Purified streptolysin, stage 5	11,100	101	111/1

This suggests that the small haemolytic activity of the unactivated streptolysin is due to the presence of minute traces of activating compounds, derived from the broth or the growth of the organism, which are only removed with difficulty.

An alternative explanation might be that, while most of the streptolysin —SH groups are comparatively rapidly autoxidizable, a remainder oxidize only very slowly. The relatively smaller 'residual' activity of the purified haemolysin might simply indicate more complete oxidation due to the amount of manipulation it has undergone in purification. This argument is strengthened by the fact that oxidizing agents such as —S—S— compounds and H_2O_2 greatly diminish this 'residual' activity as shown in Table 2.

Yet a third explanation, that the residual haemolysis is due to another, different, haemolysin, does not seem probable, since it is completely abolished by anti-streptolysin O and by the θ antiserum to *Cl. welchii*.

The results so far described are mostly in good agreement with the thiol-disulphide hypothesis outlined above; in spite of its attractive nature, however, this hypothesis can by no means be taken for granted, as later results will show.

(*d*) *Effect of* p*H*. Fig. 1 shows the effect of carrying out tests of haemolytic activity in a series of isotonic buffers of varying *p*H. The haemolytic activity is greatest at *p*H 6·5 and falls off markedly on both sides of this 'optimum *p*H'; the curve, in fact, resembles the *p*H-activity curve of an enzyme. The two results at *p*H 8·0 and 8·05 show that there is no significant difference between borate and phosphate buffers of the same *p*H.

It is of course impossible to say whether the effect of *p*H is on the haemolysin or the red cells or both. These experiments do show, however, the necessity of conducting haemolysis tests in buffered media if accurate results are to be obtained.

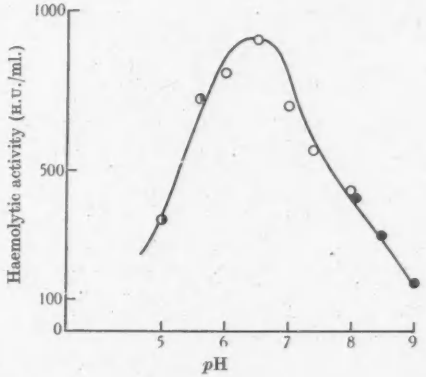


Fig. 1. Effect of *p*H on haemolytic activity. ◑ phosphate-citrate buffer. ○ phosphate buffer. ● borate buffer.

(*e*) *Effect of temperature.* The haemolytic activity of the same streptolysin solution was tested at 0, 18 and 38°. The results are shown in Table 3. The haemolytic activity is very much reduced at 0°, being only 3 % of the activity

Table 3. *Effect of temperature*

Purified streptolysin activated with thiolacetic acid was used. Varying amounts were measured into small tubes, brought to 1 ml. and these and the red cell suspension separately brought to the required temperature before mixing. Incubated 30 min. in each case.

Temp.	Haemolytic activity (H.U./ml.)	Relative activity %
0°	19	3·2
18°	234	39·9
38°	588	100

at 38°; the activity at 18°, however, is 40 % of the activity at 38°. In other words the haemolytic process appears to have a temperature coefficient which decreases with temperature.

Certain bacterial haemolysins, e.g. the α-haemolysin of *Cl. welchii* [van Heyningen, 1941] and staphylococcus β-haemolysin, display the phenomenon of so-called 'hot-cold' lysis; that is, if an appropriate amount of haemolysin is incubated with red cells at 38°, little or no haemolysis is observed, but on subsequently cooling, the red cells are haemolysed. It may be worth recording that streptolysin O does not display this interesting though somewhat obscure phenomenon.

(*f*) *Adsorption of the haemolysin on the red cell surface.* As shown above, when moderate amounts of streptolysin are mixed with red cells at 0°, no haemolysis occurs in half an hour. If the red cells are then centrifuged, all the haemolysin has disappeared from the supernatant, as can be seen by adding fresh red cells

to it and incubating at 38°, when no haemolysis occurs. The haemolysin has all been adsorbed on the original red cells, as can be shown by suspending them in fresh saline and incubating at 38°, when they are rapidly haemolysed. Small amounts of streptolysin are all adsorbed on the red cells as described; with larger amounts the red cell surface becomes 'saturated' and some haemolysin remains unabsorbed (Table 4).

Table 4. *Adsorption of streptolysin O on red cells at 0°*

Varying amounts of a streptolysin O solution activated with $Na_2S_2O_4$ were incubated with 1 ml. portions of red cell suspension for 30 min. at 38°, in a total volume of 2 ml. The following results were obtained:

Streptolysin (ml.)	0·2	0·1	0·05	0·02	0·01
Haemolysis (%)	100	100	100	60	10

A similar series of tubes of the same streptolysin solution was set up at 0° (lysin and red cells being separately cooled to 0° before mixing), kept at 0° for half an hour and centrifuged. There was no haemolysis in any of the tubes. The supernatants were then drawn off and the red cells suspended in fresh saline; the supernatants were added to fresh red cells. Both series of tubes were incubated 30 min. at 38°, with the following results:

Streptolysin (ml.)	0·2	0·1	0·05	0·02	0·01
Supernatants + fresh red cells, % haemolysis	15	5	0	0	0
Red cells + fresh saline, % haemolysis	100	100	100	60	10

(N.B. The values for percentage haemolysis were obtained by visual comparison with standards consisting of varying amounts of red cells laked by saponin; they are approximate only.)

These results show that the adsorption of streptolysin on the red cell surface is a different reaction from the actual process of lysis, in which a chemical process is probably involved, since it is inhibited, while adsorption processes are known to be increased, by low temperatures.

If an experiment similar to that in Table 4 is carried out with unactivated streptolysin, this is not adsorbed on the red cells; it all remains in the supernatant where its presence can be detected by adding cysteine followed by fresh red cells. A similar phenomenon was observed by Cohen & Shwachman [1937] with pneumolysin, which is similar to streptolysin O in many respects.

(*g*) *The effect of varying the red cell concentration.* In the course of working out the method used for the quantitative estimation of haemolysis described in the preceding paper, experiments were made with red cell suspensions of varying strengths. The results are shown in Fig. 2. The somewhat surprising phenomenon was observed that when small amounts of haemolysin were added, so that only a small percentage of the red cells present were haemolysed, the amount of haemolysis was greater the smaller the number of red cells present. A tentative explanation of this phenomenon is that (*a*) once streptolysin is adsorbed on the red cell surface it is not easily released, (*b*) for each individual red cell, a certain critical amount of streptolysin must be adsorbed on its surface to cause haemolysis in a given time, and if less than this amount is present no haemolysis takes place (*vide* the preceding paper). Hence with a large amount of red cells and a small amount of haemolysin, the latter may be so 'spread out' over the total red cell surface that there is insufficient on any one red cell to cause haemolysis. It may be for this reason that, when measuring haemolysis, irregular results are

obtained if the haemolysin and red cells are not properly mixed at the moment of addition.

However that may be, these results show that haemolysis tests can only be accurate if red cell suspensions of exactly known strength are employed.

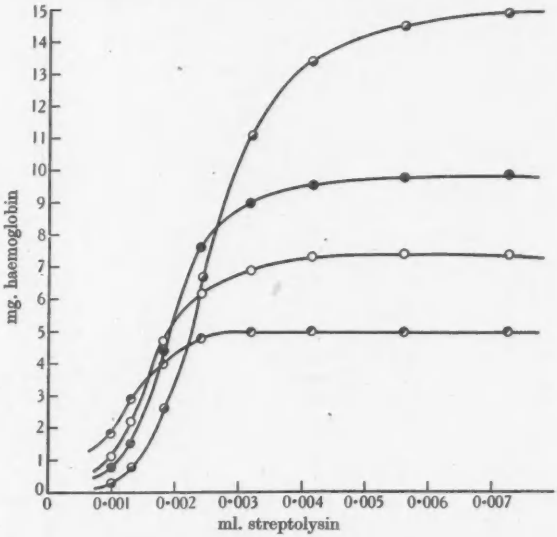


Fig. 2. Effect of varying red cell concentration. The four curves represent four series of measurements with red cell suspensions containing 15, 10, 7·5 and 5 mg. haemoglobin per ml.

(*h*) *The effect of various substances on the haemolysin.* The α-haemolysin (or α-toxin) of group A *Cl. welchii* has been shown by Macfarlane, R. G. *et al.* [1941] to be activated by calcium and to be inhibited by substances which combine with ionic calcium such as phosphate, citrate etc. Experiments were conducted to determine whether the same holds for streptolysin O.

It has already been shown that phosphate has no inhibiting action, since the haemolytic activity is the same in phosphate and borate buffers of the same *p*H (Fig. 1). Table 5 shows that fluoride, oxalate and citrate also have no effect. Likewise the addition of calcium to streptolysin preparations (exhaustively dialysed against glass-distilled water and presumably calcium-free) causes no increase in haemolytic activity, but a small decrease. The addition of magnesium also has no effect.

It has been claimed that sulphanilamide inhibits streptolysin. To test this, 20 mg. sulphanilamide were added to 2 ml. of a streptolysin O solution, followed after 20 min. by 0·5 ml. of *M*/25 thiolacetate; the mixture was then diluted and tested in the usual way, a control being run simultaneously, with the following results:

	H.U./ml.	Inhibition %
Control	182	—
Sulphanilamide	159	13

The result can hardly be considered significant.

Table 5. *Effect of calcium, etc.*

Streptolysin O activated with thiolacetic acid was used. The red cells were suspended in 0·9% NaCl and all dilutions of the streptolysin were made in 0·9% NaCl containing the various substances adjusted to *p*H 6·5, instead of the usual saline-phosphate.

Substance added	Haemolytic activity H.U./ml.	Inhibition %
Exp. 1		
None (control)	801	—
$M/1000$ $CaCl_2$	596	25
$M/50$ NaF	775	3
$M/100$ potassium oxalate	800	0
$M/100$ sodium citrate	790	2
Exp. 2		
None (control)	575	—
$M/100$ $CaCl_2$	319	45
$M/100$ $MgCl_2$	578	0

N.B. The final concentrations of the various substances are given; i.e. after the streptolysin and red cells have been mixed. The two experiments were carried out with different streptolysin preparations.

The effect of iodoacetic acid and iodoacetamide, was considered interesting, since many enzymes are known which are activated by —SH compounds (papain, cathepsin, succinic dehydrogenase etc.) in a similar way to streptolysin O, and all such enzymes are strongly and irreversibly inhibited by iodoacetic acid or its amide, often in concentrations as low as $M/1000$. However, Table 6

Table 6. *Iodoacetic acid and iodoacetamide*

Exp. I. 1·0 ml. streptolysin O + 1·0 ml. iodoacetic acid ($M/25$) kept 30 min. at room temperature; 1·0 ml. $M/5$ thiolacetic acid then added and after 10 min., diluted and measured haemolytic activity.

	Haemolytic activity H.U./ml.
Control	851
Iodoacetic acid	862

Exp. II. As exp. I, but the streptolysin O was incubated with the iodoacetic acid for 20 min. at 38°; similarly with iodoacetamide.

	Haemolytic activity H.U./ml.	Inhibition %
Control	524	—
Iodoacetic acid	406	22
Iodoacetamide	207	60

shows that even $M/50$ iodoacetic acid has no effect on streptolysin O when kept with it for 20 min. at room temperature and only a slight effect at 38°. Even $M/50$ iodoacetamide, which is considered to react with protein —SH groups more readily than iodoacetic acid, only causes partial inhibition. These results are in complete agreement with those of Symthe & Harris [1940], and as these authors state, are not in agreement with the hypothesis that streptolysin O activity depends on a thiol-disulphide system.

Nevertheless, it might be argued for this hypothesis that in these experiments iodoacetic acid was added to the inactive streptolysin presumably in the disulphide state, and that iodoacetic does not attack —S—S— links.

To test this, excess of iodoacetic acid was allowed to act upon streptolysin activated with thiolacetic acid and hence presumably in the —SH state. Twice as much iodoacetic acid was added as was needed to combine with all the thiolacetic acid present. On testing again, it was found that the haemolytic activity had disappeared. This inactivation was partly reversible, however, and on adding a further excess of thiolacetic acid, the haemolytic activity was restored to the extent of 40 %. The actual figures were as follows:

	Haemolytic activity H.U./ml.
(1) Unactivated streptolysin	18
(2) Streptolysin + thiolacetic acid (M/50)	870
(3) Streptolysin-thiolacetic acid + iodoacetic acid (M/25) for 30 min.	23
(4) Above + thiolacetic acid (M/10)	360

This seems to indicate either that the activity of streptolysin O is not dependent upon reduced —SH groups, or that the —SH groups of this compound react very slowly with iodoacetic acid. These results also are in agreement with those of Smythe & Harris.

Many other substances that oxidize or combine with —SH groups will bring about reversible inhibition of activated streptolysin, e.g.

	Haemolytic activity H.U./ml.
(1) Unactivated streptolysin	19
(2) Streptolysin + thiolacetic acid (M/50)	866
(3) Streptolysin-thiolacetic acid + H_2O_2 (M/50) for 30 min.	4
(4) Above + thiolacetic acid (M/25)	790

(*i*) *Serological properties.* Todd [1934] showed that tetanolysin and pneumolysin were both neutralized by anti-streptolysin O, though to a less extent than streptolysin O. The conclusion is that these three haemolysins are chemically very similar substances, so much so that they are all neutralized by the antiserum to any one of them. The work of Shwachman *et al.* [1934] confirms this view since it shows pneumolysin to resemble streptolysin O in many ways, including the phenomenon of activation by —SH compounds. Todd [1941] and van Heyningen [1941] have shown that one of the haemolysins of *Cl. welchii*, the θ-haemolysin, also resembles streptolysin O in being activated by $Na_2S_2O_4$, —SH compounds etc., and Todd has shown that here also the phenomenon of cross-neutralization occurs, *welchii* θ-haemolysin being neutralized by anti-streptolysin and *vice versa*.

Table 7 shows that purified preparations of streptolysin O are neutralized by anti-streptolysin O to the same extent as crude preparations; also that they are neutralized by antisera to *Cl. welchii* θ-haemolysin, but not by antisera to *welchii* α-haemolysin. The fact that crude and purified preparations are neutralized to the same extent shows that little conversion into toxoid occurs during the purification process (if, indeed, streptolysin O can exist in a toxoid form, which is not known).

The α-haemolysin of *Cl. welchii* is probably a lecithinase [Macfarlane, M. G. & Knight, 1941] and has the property of producing a flocculent precipitate when added to a solution of lecitho-vitellin prepared from egg-yolk [Macfarlane, R. G. *et al.* 1941]. Streptolysin O does not give this reaction, no trace of opalescence being observed after incubating a strong solution of streptolysin (1850 H.U./ml.) in 0·9 % NaCl for 24 hr. at 38° with the egg solution.

Table 7. *Neutralization of streptolysin by anti-streptolysin O and by antisera to* Cl. welchii *toxins*

The streptolysin O solutions activated with thiolacetic acid were titrated, and amounts corresponding to exactly 10 H.U. measured out into a series of tubes containing varying amounts of anti-sera, made up to 1 ml. After 15 min. at room temperature 1 ml. of red cell suspension was added and the tubes incubated for 30 min. at 38°. Below are given the amounts of the various sera required for complete neutralization of 10 H.U. The streptolysin O broth used had 15·3 H.U./mg. dry weight; the purified preparation, which was made from the same sample of broth, had 780 H.U./mg.

Serum	Amount of serum required to neutralize 10 H.U. of streptolysin O: Purified streptolysin ml.	Crude streptolysin broth ml.
(1) Anti-streptolysin O	0·0056	0·0049
(2) *Cl. welchii* θ anti-toxin	0·00049	0·00042
(3) *Cl. welchii* α anti-toxin	0·27	0·21

Serum (2) containing 100 θ and 0·2 α units per ml., and serum (3) containing 100 α and 0·3 θ units per ml., were kindly provided by Dr Glenny.

(*j*) *Lethal effect on mice.* Purified streptolysin O preparations are toxic to mice on intravenous injection. The minimum lethal dose was about 135 H.U. or 0·044 mg. of the purest preparation. The streptolysin O has a much greater lethal effect when injected in an activated form, e.g. together with cysteine, although the cysteine alone has no harmful effect (Table 8).

This suggests that *in vivo* its action is similar to that *in vitro*; it also suggests that the lethal effect is due to streptolysin O itself and not to some impurity.

Table 8. *Lethal effect of streptolysin O*

Purified streptolysin O solutions in 0·9% NaCl were injected into the tail veins of albino mice (*ca.* 20 g.). The volume injected was always made up to 0·5 ml.

	Streptolysin injected mg.	Total H.U. injected	No. mice injected	Deaths	Interval till death
		Exp. 1			
(*a*) Streptolysin activated with cysteine	0·176	540	2	2	30 sec.; 30 sec.
	0·088	270	2	2	30 sec.; 3 min.
	0·044	135	2	1	40 min.
	0·022	67·5	2	0	Survived
(*b*) Unactivated streptolysin	0·352	1080	2	1	Died overnight
	0·176	540	2	1	,,
	0·088	270	2	0	Survived
	0·044	135	2	0	,,
		Exp. 2			
(*a*) Streptolysin activated with cysteine	0·242	740	2	2	10 sec.; 10 sec.
	0·098	296	2	2	15 sec.; 20 sec.
	0·048	148	2	1	7 min.
	0·024	74	2	0	Survived
(*b*) Unactivated streptolysin	0·240	740	2	0	,,
	0·098	296	2	0	,,

As a control experiment, 3 mice were injected with 0·5 ml. *M*/25 cysteine (neutralized) in 0·9% NaCl. All survived with no apparent harmful effects.

(*k*) *Streptolysin O and other toxins of group A streptococci.* Group A haemolytic streptococci are stated in the literature to produce the following toxic substances, (*a*) the erythrogenic or scarlet fever toxin, (*b*) streptolysin O,

(*c*) streptolysin S, (*d*) a leucocidin, (*e*) a fibrinolysin, (*f*) a diffusing factor. The relations between these factors have hitherto remained obscure.

The non-identity of streptolysins O and S has already been demonstrated by Todd, and further demonstrated in the course of this work and work in progress on streptolysin S.

It may also be concluded that streptolysin O and the erythrogenic toxin are two different substances. Barron *et al.* [1941] have obtained highly purified erythrogenic toxin, and have shown it to be a protein of low molecular weight and stable to heat; its activity in the skin test is not affected by oxidizing and reducing agents; comparison of the properties of their product and ours makes it certain that the two substances are different. Todd *et al.* [1933] have already shown that the antibodies to these two toxins are different.

Dr McClean has kindly tested purified streptolysin O preparations for diffusing factor and hyaluronidase activities, and found these to be completely absent. Streptolysin O and diffusing factor are therefore different substances.

Discussion

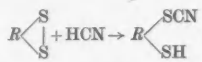
As a result of this work streptolysin O has been shown to be a protein, and highly purified preparations of it have been obtained. This protein haemolyses red blood corpuscles, but only after it has been activated with hydrosulphite, cysteine, or a number of other reducing agents. In this behaviour it resembles a number of enzymes which are similarly affected by oxidation and reduction (urease, arginase, papain, cathepsin, succinic dehydrogenase, triosephosphate dehydrogenase and several others). All these enzymes are strongly inhibited by low concentrations of iodoacetic acid.

The current theory is that these enzymes possess —SH groups capable of reversible oxidation and reduction which are essential for enzymic activity. This explains many facts including their inactivation by iodoacetic acid which is known to combine with —SH groups. Does this theory cover streptolysin O?

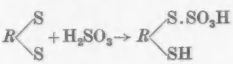
We have seen that the theory explains many of the observed facts. Moreover, purified streptolysin O preparations do contain —S—S— linkages, or at least linkages which can be converted into —SH groups by treatment with reducing agents such as KCN.

Inactive streptolysin O is not adsorbed by red blood cells, while the active form is, and it is tempting to suppose that the —SH groups are necessary to attach the haemolysin to the red cell.

There are, however, a number of facts which are not in accord with this hypothesis. One is the very feeble activation of streptolysin O by KCN, which strongly activates many of the above enzymes. This might be ascribed to the fact that the reaction of KCN with —S—S— links only releases half the available —SH groups:



Na_2SO_3, however, behaves in an entirely analogous way:



yet Na_2SO_3 activates streptolysin O as efficiently as cysteine or reduced glutathione.

The resistance of streptolysin O to inactivation by iodoacetic acid is also difficult to reconcile with the above theory.

The '—SH theory' is much in vogue at the present time, yet even in the case of certain enzymes there are many facts that conflict with it. Fruton & Bergmann [1940] completely reject this theory; they suggest that in the activation of papain, for example, cysteine, HCN and reduced glutathione do not act by reducing the —S—S— groups of papain to —SH, but act rather as 'coenzymes', forming a dissociable cysteine-papain complex, a dissociable KCN-papain complex, etc.

In the case of streptolysin O, this theory explains the observed facts at least as well as the other. It explains, for example, the inactivation of streptolysin-cysteine on dialysis and the apparent anomaly in the action of iodoacetic acid. On the theory of Fruton & Bergmann, iodoacetic acid, H_2O_2 and other oxidizing agents bring about reversible inactivation of streptolysin-cysteine, not by oxidizing the —SH groups of the streptolysin, but simply by removing the cysteine. It does not explain so well the fact that the eight different compounds mentioned in Table 2 all activate streptolysin O to exactly the same extent.

At present it is not possible to decide whether either of these theories adequately explains the activation of streptolysin O by reducing agents.

Another problem of major importance is the manner in which activated streptolysin O brings about the haemolysis of red cells. The simplest explanation is that it is an enzyme which attacks some constituent of the red cell membrane. Its protein nature, its activation by —SH compounds and many of its other properties all suggest this. So far, however, no enzymic function has been found for it; the difficulty of course lies in finding the appropriate substrate, which can only be a matter of trial and error. The exceedingly small doses in which all bacterial toxins work suggest that their action must be catalytic. Two bacterial toxins have already been identified as enzymes, namely the α toxin of *Cl. welchii*, which M. G. Macfarlane & Knight [1941] have shown to be probably a lecithinase, and the 'diffusing factor' of the same organism, and of streptococci which McClean & Hale [1941] and McClean [1941] have shown to be probably hyaluronidases. The possibility exists that all bacterial toxins are in fact enzymes, and this would seem to be a fruitful line of approach at the present time.

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128. STUDIES IN IMMUNOCHEMISTRY

6. THE USE OF PHENOL AND OF ALKALI IN THE DEGRADATION OF ANTIGENIC MATERIAL ISOLATED FROM *BACT. DYSENTERIAE* (SHIGA)[1]

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Earlier work has shown [Morgan, 1937; Morgan & Partridge, 1939, 1, 2; 1940, 1; Partridge & Morgan, 1940] that the antigenic substance which is responsible for the dominant serological properties of the 'smooth' form of *Bact. dysenteriae* (Shiga) can be extracted from the micro-organism by means of diethyleneglycol. The properties of the antigenic complex were described in some detail and three major components were isolated and identified as polysaccharide, phospholipin and a protein or polypeptide-like substance. By the action of cold, neutral formamide, the phospholipin component was removed and there remained a complex of specific polysaccharide and the polypeptide-like component which still possessed powerful antigenic properties. The material dissolved in phosphate buffer (*p*H 7·5–8·0) solution containing 0·85% NaCl to yield a clear 0·2% solution which could readily be passed through a sterile Berkefeld candle without appreciable loss. The protein component of the original antigenic complex on the other hand could be largely removed by the action of trypsin but the resulting polysaccharide-phospholipin complex possessed no significant antigenic capacity, as measured by the production of 'Shiga' agglutinins and precipitins.

A number of other experimental procedures designed to separate the components of the antigenic complex were also mentioned. The material was shown to yield clear solutions in cold anhydrous formic acid and in concentrated solutions of phenol. From these solvents the antigenic material could be largely recovered by precipitation with alcohol or acetone. Details of our work with the latter solvent are now given with some observations on the action of alkali on the antigenic complex.

Experimental

It has already been shown [Morgan & Partridge, 1940] that the protein or polypeptide-like component of the antigenic complex of *Bact. dysenteriae* (Shiga) can be removed by the action of trypsin or by the dissociating action of anhydrous formamide. The experimental work described below extends these observations and indicates that this component can also be eliminated from the antigenic complex by the action of phenol or by repeated precipitation of the complex from alkaline solution by alcohol. All these procedures, however, destroy the capacity of the resulting substance to induce the formation of 'Shiga' agglutinins and precipitins. As a result of the work to be described in this paper the so-called polypeptide-like component of the antigenic complex is now considered to be a conjugated protein and therefore the former term 'polypeptide-like' component will no longer be used.

[1] For Part 5 see Partridge & Morgan [1940].

[2] Beit Memorial Research Fellow.

The action of phenol on the antigenic complex

In some early experiments attempts were made to remove the protein component of the antigenic complex by thoroughly extracting a 2–3% aqueous solution of the primary diethyleneglycol extraction product [Morgan & Partridge, 1940, 1] with a strong solution of phenol. The upper aqueous layer was removed and again treated with liquid phenol but the product finally recovered from the aqueous layer still contained protein and represented a large proportion of the original material. Examination of the resulting product revealed, however, that the phospholipin component had been removed during the phenol extraction.

During some attempts to separate from crude preparations of the antigen, agar or agar hydrolysis products, such as kanten, by means of their insolubility in 90% liquid phenol, it was observed that the antigenic complex was readily soluble in phenol of this strength and could be recovered from phenol solution by precipitation with alcohol. At first no attempt was made to fractionate the phenol-soluble material but it was noticed that after several treatments with phenol in this manner there was a significant decrease in the nitrogen content of the product finally isolated. Examination of the successive alcoholic phenol mother liquors showed that the amino-acid complex was being gradually eliminated. It was also observed that the antigenicity of the complex recovered after phenol treatment was considerably reduced, presumably owing to the elimination of the protein, and for this reason the degradation process was not extended, at that time, to eliminate the whole of the protein component. At this stage in the investigation Palmer & Gerlough [1940] described a method whereby the 'O' antigenic complex of *Bact. typhosum* could be recovered from the dry bacterial cells or from antigenic preparations by extraction of the material with 88 or 95% liquid phenol. The antigenic complex remained insoluble in phenol of this strength. Our earlier experiments on the action of phenol on the antigenic complex of *Bact. dysenteriae* (Shiga) were continued, using a technique very similar to the one described by these authors.

Dissociation of the antigenic complex. A preparation (1·25 g.) of the antigenic complex of *Bact. dysenteriae* (Shiga), $[\alpha]_{5461}+65\pm5°$, N, 4·4%, after treatment with formamide to remove the phospholipin component, was treated with two quantities of 30 ml. of 90% phenol. The material dissolved completely and could be recovered from the combined phenol solutions by the addition of alcohol. The greater part (0·95 g.) of the dissolved substance was thrown out of solution between the alcohol levels 0 and 33% by volume. There was no precipitate formation on increasing the alcohol concentration from 33 to 50%. The alcoholic supernatant was dialysed to remove phenol and alcohol and the resulting aqueous solution was taken to dryness *in vacuo*. This fraction gave strong biuret and Sakaguchi tests. The material thrown out of 90% phenol solution between 0 and 33% alcohol levels contained 3·4% N and 3·8% ash. It was again treated with 90% phenol but the volume of phenol solution was reduced so that a small amount (5–10%) of the material was left undissolved. Once again the main portion of the substance was recovered from the phenol solution by the addition of alcohol to 33%. The material showed $[\alpha]_{5461}+68\pm4°$, N, 3·0%, P, 0·64%. The fraction was then extracted with 92% phenol (30 ml.) in an attempt to separate a larger phenol-insoluble portion. The insoluble material weighed 0·32 g. and contained 3·1% N, 0·64% total P and no inorganic P. The composition is seen to be very similar to that of the 90% phenol-soluble material which was precipitated by the addition of alcohol up to 33%. A 0·5% aqueous solution of the material insoluble in 92% phenol was too opalescent for accurate deter-

mination of specific rotation. The material precipitated from solution in 92% phenol by 33% alcohol gave the following analytical data: $[\alpha]_{5461}+72\pm3°$; N, 2·97%; P, 0·77%; inorganic P, nil. A quantitative Sakaguchi test carried out according to the method of Jorpes & Thorén [1932] showed the material to contain at least 0·15% arginine. The phenol extraction of the material insoluble in 92% phenol was repeated twice but each successive phenol-insoluble portion showed a similar rotation and N content and the Sakaguchi colour reaction indicated that in each preparation about 0·15–0·30% of arginine still remained in the phenol-insoluble fractions. The analytical figures for the material showed $[\alpha]_{5461}+76\pm4°$ (*c*, 0·5) N, 2·95%; P, 0·75%.

After the above phenol extraction experiments had been completed work on the recombination of the non-antigenic specific polysaccharide and protein components of the 'Shiga' antigenic complex [Partridge & Morgan, 1940] indicated that from solution in certain organic solvents these components could be precipitated in the form of an antigenic complex that possessed chemical, physical and immunological properties very similar to those of the original polysaccharide-protein complex derived from the native antigen. These observations suggested that the recovery of phenol-soluble and dissociated material by precipitation with alcohol caused the free polysaccharide and protein components to recombine for the most part, and thus the material precipitated from phenol solution by alcohol was composed to a considerable extent of a complex of these components and not of a simple mixture of polysaccharide and protein. The alcoholic phenol supernatant fluid, however, usually contained some of the free protein component of the antigenic complex which could be readily recovered after dialysis of the fluid to remove alcohol and phenol. The existence of the uncombined amino-acid complex in the alcoholic phenol offers an explanation for the lower protein content of the material precipitated from phenol solution by alcohol.

The experiments described above failed to show, in a clear cut manner, whether the protein component could be preferentially extracted from the antigenic complex by simple leaching with phenol solution. In order to obtain further information on this point, the experiment was repeated using a 95% phenol solution for extraction. The antigenic material was only sparingly soluble in this solvent and thus, even after several extractions with phenol, a considerable part of the complex remained undissolved and was therefore available for analysis and for antigenicity tests.

A preparation of the primary diethyleneglycol extraction product was thoroughly treated with cold anhydrous formamide (M.P. 2°) to remove the phospholipin component and, as a result of the thorough treatment, the product was found to have lost part of its polysaccharide as well as the phospholipin component. The analytical figures were 6·4% N; 1·4% P; inorganic P, nil; ash, 5·0%. A similar polysaccharide-protein complex containing a high proportion of the latter component has already been described in detail [Morgan & Partridge, 1940, 1]. The specific rotation of the material could not be determined with accuracy owing to a heavy bluish opalescence but was estimated to be $+30\pm5°$. It has frequently been observed that preparations of antigenic material possessing a high content of the protein component show an intense bluish opalescence when in aqueous alkaline solution.

The phospholipin-free material (1·0 g.) was treated with 30 ml. of 95% phenol solution and after thorough shaking was set aside for 30 min. at room temperature. The material swelled up to a thin transparent jelly but was only sparingly soluble in the solvent. The suspension was centrifuged and the

insoluble material was resuspended in fresh 95% phenol. After an hour the bulk of the preparation was again recovered and resuspended in fresh phenol solution. The process of extraction with phenol was repeated seven times. Methyl alcohol was added to each phenol supernatant fluid to make a concentration of 3% and each mixture was dialysed separately until free from phenol. The addition of alcohol produced no precipitate and was made to prevent the strong phenol solution from crystallizing during manipulation and to increase the rate of diffusion of phenol through the cellophane. Some material insoluble in dilute phenol solution separated during the later stages of dialysis and finally remained insoluble after the phenol had been completely removed. The contents of each of the cellophane bags were centrifuged separately to remove insoluble matter, the clear solutions were evaporated *in vacuo* to a small volume, frozen at $-10°$ and taken to dryness *in vacuo* from the frozen state.

Examination of unchanged antigenic material. The material (S.P.T. 12A) remaining insoluble after seven extractions of the original antigenic complex with 95% phenol was recovered by washing with alcohol to remove phenol, solution in water and reprecipitation with alcohol. The material was again dissolved in water and reprecipitated with alcohol. After drying *in vacuo*, the substance weighed 350 mg., contained 6·1% N, 1·5% P, no inorganic P and gave an opalescent solution in water. The quantitative Sakaguchi test indicated the presence of 0·7% arginine and the biuret, ninhydrin and diazobenzenesulphonic acid tests were strongly positive on 2 mg. samples of the preparation. These figures were very similar to those given by the original antigenic material and indicated that the part of the antigenic complex which had not dissolved in the 95% phenol remained essentially the same in composition as the original phospholipin-free antigenic complex. There was, therefore, no evidence that the protein component was simply admixed with the polysaccharide, but the results supported the belief that these substances were combined as a molecular complex. The material remaining insoluble in 95% phenol was tested for antigenicity in rabbits. The usual course of 3 doses of 0·05 mg. produced an immune response

Table 1. *Showing the production of 'Shiga' agglutinins in rabbits inoculated with substances derived from the phospholipin-free antigen by treatment with 95% phenol solution*

Material injected				Dilution of immune serum giving agglutination with formolized suspension of *Bact. dysenteriae* (Shiga)				
Description	Nature	Doses mg.	Rabbit no.	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
Material insoluble in 95% phenol (S.P.T. 12A)	Polysaccharide-protein complex	3 × 0·05	231	4	3	1	1	0
			232	4	4	3	2	1
			233	4	2	1	0	0
			234	2	1	1	0	0
Material soluble in 95% phenol and soluble in water after dialysis (S.P.T. 16)	Polysaccharide	6 × 0·05	250	0	0	0	0	0
			251	0	0	0	0	0
			252	0	0	0	0	0
			253	0	0	0	0	0
Material soluble in 95% phenol and insoluble in water after dialysis (S.P.T. 13)	Protein	6 × 0·05	242	0	0	0	0	0
			243	0	0	0	0	0
			244	0	0	0	0	0
			245	0	0	0	0	0

4 Complete agglutination.
3 Almost complete agglutination.
2 Incomplete agglutination.
1 Trace of agglutination.
0 No agglutination.

in each of the four animals immunized (Table 1, animals 231, 232, 233 and 234). After a further course of immunization the sera of all rabbits gave precipitation when mixed with equal volume of 1 : 100,000 dilution of the 'Shiga' specific polysaccharide.

Isolation of the polysaccharide component. The results of an examination of the phenol-soluble fraction that was also soluble in water after dialysis are given in Table 2. It appears that the water-soluble material extracted by phenol from

Table 2. *Showing the amount and composition of material extracted from the antigen of* Bact. dysenteriae (*Shiga*) *by strong solutions of phenol*

Phenol extract	Volume ml.	Weight mg.	Nitrogen %	Total* phosphorus %	Arginine %
I	30	54·2	1·15	—	—
II	30	75·3	1·68	0·55	0·05–0·1
III	30	70·3	1·78	0·74	0·05–0·1
IV and V	2 × 20	86·4	2·27	1·00	0·10
VI	30	78·4	2·38	0·87	0·07
VII	30	67·0	2·10	0·98	0·01

* Inorganic P, 0·0 %.

the antigenic complex was almost entirely polysaccharide in nature. Traces of arginine remained in the soluble material but this was to be expected in view of the very simple treatment involved. The combined extracts weighed 431·6 mg., and 5 mg. portions of the material failed to give a positive biuret, ninhydrin, α-nitroso-β-naphthol or diazobenzenesulphonic acid test. The Molisch test was strongly positive. Fractions II–VII after solution in water were combined, made distinctly acid with acetic acid, spun to remove a small amount of insoluble matter and precipitated by the addition of several volumes of alcohol. The preparation showed an opalescence which rendered the determination of specific rotation difficult. The substance contained 2·05 % N, 0·81 % P and no inorganic P. The material was tested in rabbits for antigenicity; 3 animals received 6 doses each of 0·05 mg. intravenously. There was slight production of specific agglutinins and precipitins although the immune response was negligible when compared with that elicited by the original phospholipin-free antigenic complex. The material was again dissolved in phenol solution (92 %) and after the addition of 3 % methyl alcohol, was dialysed in a cellophane bag until free from phenol. The solution was freed from a small amount of insoluble matter, concentrated to 10 ml. and taken to dryness *in vacuo*. A solution (1 %) of the substance (S.P.T. 16) possessed slight opalescence, but the material (10 mg.) gave a negative Sakaguchi test. Arginine (0·01 mg.) under the same conditions gave a definite rose-pink coloration. Analytical data: $[\alpha]_{5461} + 84 \pm 3°$ (0·5 % in H_2O); N, 1·82 %; P, 0·75 %, inorganic P, 0·0 %. The material was again tested for antigenicity in 4 rabbits; there was no detectable immune response (Table 1, animals 250, 251, 252 and 253).

Isolation of the protein component. The material which separated during dialysis of the phenol extracts (I–VII) was collected and extracted with 2 ml. *N* NaOH. The insoluble residue was again extracted with 1 ml. 0·1 *N* NaOH and finally washed with several 1 ml. quantities of distilled water. The residue appeared to be largely inorganic, weighed 60 mg. and contained 0·38 % N. The alkali-soluble material and the water washings were mixed together, acidified with acetic acid and the resulting precipitate was collected, dissolved by the addition of alkali to *p*H 8·0 and again recovered by the addition of acid. The

precipitate was thoroughly washed with 0·05 *N* acetic acid, alcohol and dried *in vacuo*. The substance (S.P.T. 13) weighed 120 mg., gave an intense biuret test and according to an arginine determination made by Jorpes & Thorén's [1932] modification of Sakaguchi's test, contained about 5 % of this amino-acid. The material gave an almost colourless solution (0·5 % in 0·1 *N* NaOH), possessed a strong negative rotation, $[\alpha]_{5461} - 60 \pm 3°$ (*c*, 1 in 0·1 *N* NaOH), contained 12·3 % N, no appreciable quantity of P, and a 5 mg. sample gave a very faint nitroprusside test for S after Na fusion. A diazobenzenesulphonic acid test on 1 mg. of the preparation gave a strong red coloration in 2–3 sec.; a control test employing the reagents alone gave a pale yellow colour only during the first 1–2 min. The fraction consists, therefore, of the protein that was originally associated with the polysaccharide that had been recovered in the phenol-soluble fractions I–VII. The results of antigenicity tests on this material are shown in Table 1 (animals 242, 243, 244 and 245); there was no detectable immune response. The figures given in Table 2 indicate that the total weight of the antigenic material dissolved in each 30 ml. of 95 % phenol used for extraction remained approximately constant and that in all 530 mg. of the antigenic complex dissolved in 190 ml. of phenol solution.

An examination of the protein isolated by this method showed that the substance was more soluble in dilute acids than samples of protein prepared by acetic acid hydrolysis of the 'Shiga' antigen and that in this property the two protein preparations appeared to be different. For further investigation a quantity of the material was therefore prepared by the following simplified process. The diethyleneglycol primary extraction product (400 mg.) was dissolved in 90 % phenol solution (20 ml.) and a small insoluble residue was removed. The clear solution was dialysed against running tap water in a cellophane bag until free from phenol and the precipitate which formed in the bag was collected. After extraction, first with alcohol and then with alcohol-ether mixture, to remove phospholipin, the material was dissolved in dilute NaOH and the small insoluble residue was removed by centrifuging. The substance was then subjected to three successive precipitations from dilute alkaline solution with acetic acid at *p*H 4·6, the protein thrown out of solution being redissolved each time in water by the addition of a minimum quantity of dilute NaOH. It was then noticed that the protein exhibited a marked solubility in *N* acetic acid and in HCl at about *p*H 2. Addition of further HCl, however, produced a heavy precipitate presumably due to the production of an insoluble hydrochloride. The protein was further purified by precipitating twice from an alkaline solution with HCl to make a final concentration of *N* HCl. The insoluble hydrochloride was washed with absolute alcohol to remove the excess of HCl, and the substance was then found to dissolve in distilled water to yield a clear colourless solution showing an acid reaction. The protein was finally precipitated from solution by the addition of sufficient solid sodium acetate to raise the *p*H to about 4·5. The protein component recovered in this manner was found to be free from P, showed $[\alpha]_{5461} - 74 \pm 2°$ (*c*, 1 in 0·1 *N* NaOH) and contained 13·0 % N. The protein could be precipitated from solution in *N* acetic acid by means of trichloroacetic acid, metaphosphoric acid, salicylsulphonic acid and other protein precipitants. Addition of common salt or other soluble chlorides, evoked the precipitation of the insoluble hydrochloride.

The analytical figures which we have previously given for the 'protein' component obtained from the diethyleneglycol primary extraction product by acid hydrolysis are significantly different from those obtained for the phenol-dissociated protein component. The former substance could be reprecipitated

many times from variable concentrations of the acid without appreciable loss, whereas the phenol-dissociated protein was readily soluble at about *p*H 2·5. It is apparent, therefore, that the proteins isolated from the same antigenic material by two different methods are substantially different in character and it appears that one product is most probably a degradation product of the other. The possibility that denaturation might be responsible for the difference was first considered, but the hypothesis appeared unlikely in view of the considerable difference in the analytical figures. Furthermore, it was found that the protein prepared by the phenol method, when dissolved to yield a clear solution in *N* acetic acid, could be heated for many hours at 95° without causing any precipitation to occur. The following experiment, however, indicates that the simple protein obtained by the phenol dissociation of the antigenic complex, is a derivative of the conjugated protein which is obtained by heating the antigenic complex with dilute acetic acid.

Isolation of the simple protein from the conjugated protein

A sample of the crude conjugated protein, prepared by hydrolysis of the antigenic complex with dilute acetic acid according to the usual method, was freed from traces of fatty impurities by extraction with an alcohol-ether mixture and was subsequently precipitated from faintly alkaline solution with acetic acid. The procedure was designed to limit the manipulations to the minimum consistent with the isolation of an apparently homogeneous material. The substance contained 11·2 % N, 1·07 % P, and possessed $[\alpha]_{5461} - 48 \pm 3°$ (*c*, in 0·1 *N* NaOH). The material was not soluble in *N* acetic acid, even on warming. The conjugated protein (400 mg.) thus obtained dissolved readily in 90 % phenol solution (10 ml.) and was largely thrown out of solution by the addition of ethyl alcohol (10 ml.). The precipitate was redissolved in phenol (10 ml.), again thrown out of solution by the addition of an equal volume of absolute alcohol, thoroughly washed with alcohol and finally dissolved in water by the addition of a few drops of NaOH solution. A small insoluble residue was removed and an equal volume of 2 *N* HCl was added to precipitate the protein from solution. The precipitate was washed with alcohol to remove the excess of acid. On addition of distilled water the precipitate slowly dissolved to a rather opalescent solution. The protein was again recovered by addition of sodium acetate to reduce the acidity of the solution to *p*H 4·5. After washing with alcohol the protein was taken up in 90 % phenol, a small fraction which remained undissolved was removed and the clear supernatant fluid was precipitated by addition of ethyl alcohol to 50 % concentration. The protein was once again precipitated from phenol solution by alcohol and again recovered from aqueous solution. The protein was then found to dissolve completely in *N* acetic acid on warming (35–40°) to yield a clear colourless solution. The protein was finally recovered as the insoluble hydrochloride. After washing with alcohol the hydrochloride yielded a clear solution of acid reaction on addition of distilled water, from which it was precipitated at *p*H 4·5 by the addition of sodium acetate. The material recovered (120 mg.) contained 13·8 % N, no P, and showed a specific rotation $[\alpha]_{5461} - 88 \pm 2°$ (1 % solution in 0·1 *N* NaOH). The analytical figures and general properties of the purified protein are distinctly different from those of the starting material, but show a close agreement with those of the substance prepared by phenol dissociation of the whole antigenic complex. It is not considered that either of the preparations isolated in this manner are pure but the simple amphoteric protein has been obtained in larger quantities by other methods and is described in more detail later in this paper.

Owing to the difficulty of establishing the purity of the conjugated protein it is not clear whether all the changes in the analytical data that accompany the changed solubility properties of the protein during the rather long series of manipulations described above can be correlated with the removal of a single chemical component that might be termed the prosthetic group. In previous purification procedures the conjugated protein was dissolved in a small volume of cold 0·1 *N* NaOH and reprecipitated with dilute HCl. This procedure was usually carried out five or six times and the substance was sometimes further purified by precipitation as sodium salt by the addition of alcohol to the alkaline solution of the material. Isolated in this manner, the protein preparations contained 11–12 % N and showed $[\alpha]_{5461}$ −45 to −50°. The P content fell steadily during purification and the final product usually contained not more than 0·2 % P. From these observations it appears that the P of the protein component obtained by acetic acid hydrolysis of the antigenic complex can be largely removed without causing a significant increase in the specific rotation or nitrogen content of the protein substance. On the other hand repeated phenol treatment of the protein component of the antigen liberated by acetic acid hydrolysis not only removes completely any P present but also causes the resulting protein to possess a higher laevorotation (−82 ± 5°), a higher N content (13·5–14·0 %) and to show a changed solubility at *p*H 2·0–2·5. The influence of these changes on the immunological properties of the preparations will be described later in the paper.

Extraction of Bact. dysenteriae (*Shiga*) *with 90 % phenol solution*

In view of the marked solubility of the 'Shiga' antigenic material in 90 % phenol solution, and the observed insolubility of agar and of nucleic acid in this solvent, it was decided to attempt a phenol extraction, in the first instance, of a sample of organisms that had already been repeatedly extracted with diethyleneglycol, but which contained a further quantity of antigenic material. Organisms that had already been in contact with anhydrous diethyleneglycol at 0–2° for many months and which had been repeatedly extracted with fresh diethyleneglycol and had yielded about 7 % of their dry weight as antigenic material were observed to have remained morphologically intact.

The heavy paste of organisms collected from the bowl of the Sharples supercentrifuge after removing the excess of diethyleneglycol was repeatedly triturated with acetone, washed with this solvent and dried *in vacuo*. The fine white powder (132 g.) so obtained was shaken with 90 % phenol solution (800 ml.) and after standing for 96 hr. with occasional shaking, the bacterial residue was again collected in a Sharples centrifuge. The clear supernatant was passed through a Doulton filter candle and mixed with 3 vol. of alcohol. The precipitate which formed was then suspended in water and dialysed against water in a cellophane bag until free from phenol. The bag finally contained a heavy white precipitate and a strongly opalescent supernatant solution. The precipitate was removed by centrifuging, dried after washing with water and alcohol and weighed. The supernatant was evaporated to dryness *in vacuo* from the frozen state. Table 4 shows the yields obtained with six successive extractions of the bacteria with 90 % phenol. As the extraction proceeded the organisms disintegrated until finally the bacterial residue appeared as a clear gelatinous mass.

The material soluble in water

The white fibrous material prepared by drying the supernatant fluid from the dialysis bags, dissolved readily in water to yield heavily opalescent solutions. The extracts were pooled, suspended in water to yield a 1 % solution and the

Table 3. *Showing the production of 'Shiga' agglutinins and precipitins in rabbits inoculated with substances isolated from the antigen of* Bact. dysenteriae (*Shiga*)

Material used as antigen	No. of doses	Total antigen injected mg.	Animal no.	Precipitation test: Dilutions of polysaccharide 1 : 10^4	1 : 10^5	1 : 10^6	Agglutination test*: Dilutions of immune serum 1 : 10	1 : 20	1 : 50	1 : 100	1 : 200	1 : 500
Phenol-extracted antigen	3	0·15	228	4	4	1	4	4	4	4	4	0
			229	3	2	1	4	4	3	2	0	0
	3	0·08	253	4	4	2	4	4	4	4	4	3
			256	4	4	2	4	4	4	4	4	3
Undegraded polysaccharide prepared by phenol method (preparation S 27)	6	0·30	238	0†	0	0	0	0	0	0	—	—
			239				0	0	0	0	—	—
			240				0	0	0	0	—	—
			241				0	0	0	0	—	—
Undegraded polysaccharide prepared by alkali method (preparation S 56)	6	0·30	200	0†	0	0	0	0	0	0	—	—
			201				0	0	0	0	—	—
			202				0	0	0	0	—	—
			203				0	0	0	0	—	—
'Shiga' conjugated protein	3	0·15	243	0	0	0	0	0	0	0	—	—
			248	0	0	0	0	0	0	0	—	—
			257	0	0	0	0	0	0	0	—	—
			260	0	0	0	0	0	0	0	—	—
			261	0	0	0	0	0	0	0	—	—
Conjugated protein combined with polysaccharide S 27	3	0·08	237	3	4	1	4	4	4	4	4	3
			238	3	4	1	4	4	4	4	4	3
			239	3	4	1	4	4	4	4	4	3
Conjugated protein combined with polysaccharide S 56	3	0·08	249	1	3	1	4	4	4	4	2	0
			250	1	2	1	4	4	4	4	3	1
			251	0	1	1	4	4	3	2	0	0
Electrophoretically pure 'Shiga' simple protein combined with polysaccharide S 50	3	0·08	257	0	0	0	0	0	0	0	—	—
			260	0	0	0	0	0	0	0	—	—
			261	0	0	0	0	0	0	0	—	—

* Formolized whole culture of 'smooth' *Bact. dysenteriae* (Shiga).
† A pooled sample only was tested.

Table 4. *Material extractable with 90 % phenol from 'smooth'* Bact. dysenteriae (*Shiga*)*

Extract no.	Volume of extract ml.	Water-soluble material g.	Water-insoluble material g.
1	315	3·10	3·50
2	320	2·07	2·99
3	324	1·10	2·98
4	372	0·14	1·20
5	344	0·08	0·88
6	410	—	0·46
Total weight of extract		6·49	12·01
Percentage of dry bacteria		4·9	9·1

* After previous treatment with diethyleneglycol [see Morgan, 1937].

material was precipitated with acetone. The substance thrown out of solution between the levels 50 and 65 % acetone was collected, redissolved in water and again precipitated with acetone. The material obtained in this manner represented the bulk of the substance present in the supernatant fluid from the dialysis bags. The purified material was tested in rabbits and was found to be a powerful 'Shiga' antigen. Three doses of the substance, when given intravenously at intervals of 3 days, yield strongly agglutinating sera (Table 3, animals 228, 229, 253 and 256). The antigenic substance contained 5·8 % N, and 0·80 % P. In order to show that this material was similar to the phospholipin-free diethyleneglycol primary extraction product the following experiments were carried out.

Hydrolysis with 1 % acetic acid. The antigenic material (1 g.) was dissolved in water (66 ml.) and glacial acetic acid (0·66 ml.) added. The solution was treated in exactly the same manner as for the acid hydrolysis of the primary extraction product [Morgan & Partridge, 1940, 1]. The water-insoluble precipitate (293 mg.) after extraction with ether gave an almost clear solution in dilute alkali and proved to be insoluble at all concentrations of acetic acid to $2N$. After precipitation from slightly alkaline solution (pH 8·0) by addition of acetic acid the material contained 10·8 % N, 0·8 % P and showed $[\alpha]_{5461} - 55 \pm 5°$. The substance was further purified by solution in ice-cold 0·1 N NaOH to make a 1 % solution and precipitated by the addition of alcohol to yield 80 % by vol. and a few drops of acetic acid. The substance then contained 11·2 % N and 0·65 % P, and showed $[\alpha]_{5461} - 56 \pm 5°$. The analytical figures are in good agreement with those obtained for the conjugated protein isolated in a similar manner from the diethyleneglycol primary extraction product. The substance was tested in animals for antigenicity and was found to behave like the so-called 'polypeptide' previously isolated [Partridge & Morgan, 1940]; specific *Bact. dysenteriae* (Shiga) precipitins and agglutinins were not produced but the formation of homologous precipitins was observed.

The clear supernatant fluid from the hydrolysis mixture after removal of the water-insoluble protein component was evaporated under reduced pressure to a small volume and precipitated by addition of 15 vol. of absolute alcohol to which a few crystals of sodium acetate were added to ensure complete precipitation. The material obtained weighed 505 mg. Specific precipitation with *Bact. dysenteriae* (Shiga) immune rabbit serum was given by dilutions of the substance out to $1 : 10^6$. The material gave a colour which matched closely in tint and intensity that produced by 'Shiga' specific polysaccharide when the two were compared according to the orcinol method of Sørensen & Haugaard [1933]. After

purification in the usual manner [Morgan, 1936] the polysaccharide contained 1·68 % N and showed $[\alpha]_{5461}+106\pm2°$.

Dissociation in phenol solution. The antigenic material (1 g.) was dissolved in 90 % phenol solution (33 ml.) and after removing a small residue was dialysed against frequent changes of distilled water. A heavy precipitate of material insoluble in water gradually formed during the removal of the phenol. Precipitation of the substance was complete when the phenol had been entirely eliminated. The contents of the bag were then centrifuged, the insoluble material collected, dissolved in dilute alkali and precipitated by addition of acetic acid to *p*H 4·6. The fraction weighed 290 mg. The substance formed a clear solution in *N* acetic acid and in HCl at *p*H 2·0–2·5 and thus behaved like the protein obtained in this manner from the diethyleneglycol primary extraction product. The addition of further HCl, or a soluble chloride precipitated the protein as hydrochloride. The protein contained 14·5 % N and P was absent; $[\alpha]_{5461}-78\pm5°$. The somewhat opalescent supernatant from the dialysis bag was concentrated and precipitated by addition of 4 vol. of ethyl alcohol in the presence of a little sodium acetate. The precipitate (559 mg.) proved to be largely undegraded 'Shiga' polysaccharide. The material contained 2·1 % N and 1·33 % P. The viscosity of a 0·5 % solution in 0·9 % NaCl solution was 1·32 (solvent, 1).

The above experiments show the antigenic material isolated by phenol extraction of the organism to be similar in chemical constitution and physical properties to the diethyleneglycol primary extraction product except for the expected absence of the phospholipin component.

The material insoluble in water

From the yield of this fraction obtained by phenol extraction and subsequent dialysis (Table 4) it will be seen that it represents a major constituent of the somatic substance of *Bact. dysenteriae* (Shiga). The substance dissolved, except for a small residue, in dilute NaOH, showed all the usual qualitative tests for proteins and was precipitated quantitatively from alkaline solution by addition of acetic acid to *p*H 4·6.

A sample of the protein (2·5 g.) was dissolved in dilute NaOH (40 ml.) and a fraction (0·59 g.) removed at 75 % alcohol level. This material was set on one side to be worked up later with a further batch of material. A second precipitate was obtained by increasing the alcohol concentration to 80 %. A small third fraction was recovered by neutralizing the 80 % alcoholic supernatant fluid with acetic acid. It was observed that the original substance showed a considerable solubility in 90 % alcohol in the presence of alkali or of excess acid, especially when salts were present. The second and third fractions were mixed, dissolved in a few ml. dilute NaOH and yielded a perfectly clear, colourless solution. The protein was finally precipitated as the hydrochloride by the addition of HCl to *N* concentration. The precipitate dissolved in distilled water, presumably owing to hydrolysis of the acid salt, and the addition of solid sodium acetate to reduce the acidity of the solution to about *p*H 4 caused the material to precipitate completely. The substance (1 g.) was washed several times with alcohol and dried *in vacuo*. The purified protein was free from P and contained 13·7 % N (uncorrected for ash). Specific rotation $[\alpha]_{5461}-81\pm2°$ (in 0·1 *N* NaOH); $-79\pm2°$ (in *N* acetic acid). The protein developed no colour with Sørensen & Haugaard's orcinol reagent for the detection of carbohydrate.

Electrophoresis. A sample of the protein was studied in the Tiselius electrophoresis apparatus through the kindness of Dr R. A. Kekwick. The protein was dissolved to make a 2 % solution in phosphate buffer of *p*H 8, μ 0·1, containing

a drop of dilute NaOH and dialysed against the buffer solution at 0° and at constant volume until equilibrium was attained. The solution was then clear and colourless. The Schlieren picture showed the substance to be electrophoretically homogeneous.

Phenol extraction of a 'rough' strain of Bact. dysenteriae (*Shiga*)

The rough organisms, which remained as a glycol-moist paste (83 g.) after a diethyleneglycol extraction experiment, were taken up in 90 % phenol and treated in the same way as for the 'smooth' organisms. The clear phenol extract after dialysis against distilled water to remove phenol contained a heavy deposit of water-insoluble material. The supernatant solution after the precipitate was removed was quite clear and on evaporation to dryness was found to be practically free from dissolved material, thus confirming the absence of the specific antigen from this variant of the organism. The insoluble material appeared to be essentially protein in nature and after purification it dissolved readily to form clear solutions in dilute NaOH and also in *N* acetic acid. The material contained 13·5 % N (uncorrected for ash), no P and showed $[\alpha]_{5461} - 80°$ (*c*, 0·5). The substance appeared to be identical with the phenol-soluble amphoteric protein of the 'smooth' organism.

Dissociation of the antigenic complex by means of alkali

The amino-acid-containing component can readily be removed from the phospholipin-free antigenic complex by repeated precipitation of the material from alkaline solution by alcohol. It is essential that during the process the solution is kept as near 0° as possible since it has been found that on standing at room temperature or 37° alkaline solutions of the undegraded polysaccharide tend to lose their high viscosity and give rise to a degraded form of the specific polysaccharide. In one experiment a preparation (0·75 g.) of phospholipin-free antigenic material ($[\alpha]_{5461} + 65 \pm 5°$; N, 4·4 %) was dissolved in distilled water (75 ml.) cooled to 0° and NaOH cautiously added to make a 0·05*N* solution. Cold alcohol (300 ml.) was then added slowly with vigorous shaking and the solution was left at 0° for an hour. The precipitate was collected, thoroughly dialysed at 0° and the resulting solution dried *in vacuo* from the frozen state. The preparation showed $[\alpha]_{5461} + 79 \pm 3°$; N, 3·5 %. The alkaline alcoholic supernatant was acidified with acetic acid, concentrated *in vacuo*, dialysed and taken to dryness. The material obtained showed a negative rotation and gave a strong biuret test for protein. It was not further purified but was put aside to be mixed with similar fractions obtained from the subsequent alkaline supernatant fluids.

The main fraction that was precipitated by 80 % alcohol was again dissolved in water at 0° and the alkaline precipitation process was repeated. The N content of the material (0·43 g.) again fell and the specific rotation increased to $+82 \pm 5°$. A further alkaline treatment gave a product showing $[\alpha]_{5461} + 84 \pm 5°$; N, 2·2 %; P, 0·56 %. Arginine determined by Sakaguchi's method was 0·05 % and the biuret test was negative on 10 mg. of the substance. Two further precipitations from alkaline solution by alcohol gave a product that showed $[\alpha]_{5461} + 84 \pm 2°$; N, 2·07 %; P, 0·57 %. The material dissolved in water to give a clear viscous solution. A 0·5 % solution in 0·9 % NaCl gave a viscosity at 37° of 1·96 compared with the solvent as unity (Ostwald viscosimeter). The substance was given intravenously to a group of eight rabbits. Six doses of 0·05 mg. at 2–3 day intervals gave rise to no demonstrable 'Shiga' agglutinins or precipitins in any of the animals immunized whereas the original phospholipin-free complex before

alkaline treatment induced the formation of high titre agglutinins and precipitins when given in similar doses. The results obtained with four only of the rabbits immunized are shown in Table 3 (animals 200, 201, 202 and 203).

The protein component recovered by acidifying the alkaline alcoholic supernatant liquids was freed from admixed polysaccharide by repeated precipitations from alkaline solution by the addition of acetic acid to *p*H 4·5. On warming gently, the precipitate dissolved to yield a clear, colourless solution in *N* acetic acid. The protein was recovered from the acetic acid solution by the addition of NaOH to bring the solution to *p*H 4·5, and, after washing the precipitated protein thoroughly with alcohol to remove sodium acetate, was found to contain 13·8 % N. The substance was free from P and showed $[\alpha]_{5461} - 74 \pm 4°$. The analytical figures are similar to those for the simple protein prepared by phenol dissociation of the antigenic complex and indicate that the protein component of the antigen is obtained as a result of dissociation in alkaline alcoholic solution, largely in the amphoteric form.

A preparation of antigenic material that had been obtained by phenol extraction of a dry culture of *Bact. dysenteriae* (Shiga) was separated into its components by fractionation from alkaline solution as described above. The material (2 g.) was dissolved in 100 ml. of ice-cold water and made *N* with NaOH. Alcohol (200 ml.) at $-10°$ was then slowly added with vigorous shaking, the solution was allowed to stand at 0° for about 30 min., and the precipitate was collected and redissolved in 100 ml. of cold water. The process of precipitation from alkaline solution was repeated twice, the combined alkaline-alcoholic solutions being acidified with acetic acid and the protein component, which was thrown out of solution, was recovered in the usual manner. The polysaccharide finally isolated analysed as follows: $[\alpha]_{5461} + 85 \pm 2°$ (*c*, 0·5); N, 2·01 %; P, 0·55 %, inorganic P, nil. The viscosity of a 0·5 % solution in 0·85 NaCl was 1·36 relative to 0·85 % saline as 1 (Ostwald viscosimeter). The protein component showed $[\alpha]_{5461} - 80 \pm 3°$ (*c*, 0·5 % in 0·1*N* NaOH); N, 13·9 %; P, nil; and arginine estimated by Sakaguchi's method as modified by Jorpes & Thorén [1932], 6·8 %. The analytical figures obtained show that the antigenic material extracted from *Bact. dysenteriae* (Shiga) by phenol contains the same polysaccharide and protein components as the antigenic complex extracted from the organisms by diethyleneglycol.

In a series of experiments the reaction conditions were varied in order that some estimate might be made of the most suitable conditions to effect the maximum dissociation. A preparation of phospholipin-free antigenic material was dissolved in water and NaOH added to a final concentration of 0·1 *N*. After standing at 20° for 1 hr. the solution was treated with alcohol to make a final concentration of 75 % by vol. The precipitate, which contained most of the polysaccharide present, represented 77 % of the material used and contained 4·1 % N. The protein recovered from the 75 % alcoholic solution contained 11·5 % N and represented 12·5 % of the complex. The experiment was then repeated, but alcohol was added immediately after the addition of the alkali, so that the latter was only in contact with the complex for 10–15 sec. The distribution of the polysaccharide and protein components was found not to be significantly different from that observed in the previous experiment. The material precipitated by 75 % alcohol represented about 75 % of the complex and contained 3·7 % N while the crude protein recovered from the supernatant contained 10·9 % N and represented 20 % of the starting material. From these results it appears that the separation of the components from an alkaline solution of the antigenic material is a process that depends upon dissociation of the complex at high *p*H values and is not due to alkaline hydrolysis which would presumably depend largely upon

the time during which the alkali is in contact with the complex. In all experiments, repeated fractionation from alkaline solution has been necessary to produce a sample of polysaccharide uncontaminated with protein.

An experiment was also undertaken in order to form a rough estimate of the minimum conditions of alkalinity required to produce a useful degree of dissociation. The antigenic material was made up to a concentration of 1% in (*a*) 10% potassium acetate solution, and (*b*) 0·1 *N* ammonia solution (*p*H 11·0) and after a few minutes alcohol was added to make a final concentration of 75% by vol. The thoroughly washed precipitates contained 5·5% and 5·3% N respectively and in each instance practically the whole of the material was recovered as precipitate. Acidification of the alcoholic filtrate yielded no appreciable precipitate of protein. It appears, therefore, that strong alkali is essential in order to induce a degree of dissociation sufficiently marked to be of use for the purpose of separating the constituents by alcoholic fractionation.

The formation of complexes between the specific polysaccharide of Bact. dysenteriae (*Shiga*) *and proteins*

It has already been mentioned that degradation of the 'Shiga' polysaccharide takes place readily in the presence of strong alkali at 37°, or in neutral or acid solution at higher temperatures, the result being a reduction in the viscosity of the aqueous solution, and it was subsequently found that the degraded polysaccharide no longer possesses the capacity to form soluble complexes with proteins.

The undegraded specific polysaccharide, on the other hand, prepared by phenol or alkali dissociation of the antigenic substance forms acid-soluble complexes with proteins that are themselves, like the 'Shiga' protein component, normally insoluble in aqueous solution at acid reaction. If a solution of the specific polysaccharide and an alkaline solution of a protein are mixed and acidified with acetic acid a soluble, stable complex is formed and the protein is found to be no longer precipitated by the addition of trichloroacetic acid or the other acid protein precipitants, provided that an excess of the protein above that found in the native antigenic complex has not been used.

In one experiment a number of complexes of various proteins with the undegraded polysaccharide prepared by phenol dissociation of the antigenic substance were formed as follows. The protein, 0·5 ml. of a 1% solution in 0·1 *N* NaOH, was added to 2 ml. of an 0·5% solution of the specific polysaccharide. The mixture was kept for 5–10 min. at 25–30° and acidified to *p*H 4·5 by cautious addition of 0·1 *N* acetic acid. The proteins employed were hen's egg vitellin, stromatin, prepared from human erythrocytes according to the method of Jorpes [1932], electrophoretically pure human serum-γ-globulin, 'Shiga' conjugated protein and the simple protein prepared from it by phenol treatment. In each instance the solution developed a bluish opalescence on acidification, but no precipitate was formed on the addition of 10% trichloroacetic acid solution (2 ml.). Control experiments were made in which the 'Shiga' polysaccharide was replaced by gum acacia, gum tragacanth and a sample of degraded (low viscosity) 'Shiga' polysaccharide, but in each instance a heavy precipitate of protein appeared on acidification of the mixed protein-polysaccharide solution with acetic acid or trichloroacetic acid. If the protein content of the alkaline mixture of the undegraded specific polysaccharide and any of the proteins mentioned was raised above 30% a considerable opalescence developed upon acidification, while a large protein excess in the mixture gave rise to a precipitate on addition of trichloroacetic acid.

Although the complexes formed by the undegraded polysaccharide with all the proteins investigated are alike in their behaviour with protein precipitants and in the appearance of their aqueous solutions, a considerable variation in their stability toward heat treatment was noticed. The complex formed from the polysaccharide and 'Shiga' conjugated protein behaves, on heating in acetate buffer, in a similar way to the antigenic diethyleneglycol primary extraction product [Morgan & Partridge, 1940, 1], that is to say, it withstands heating to 100° at *p*H 4·6 for 2–3 hr. before flocculation occurs and the conjugated protein is thrown out of solution. Under these experimental conditions the polysaccharide itself becomes degraded and loses its power to associate with the protein. On the other hand, the complexes formed with the other proteins mentioned, including the simple amphoteric protein derived from the 'Shiga' antigen or from the organism itself, are heat-labile. On raising the temperature of a solution of the complexes in acetate buffer at *p*H 4·6 by immersion in a boiling water bath, flocculation commences almost at once and is complete in 4–5 min., the protein separating as an insoluble precipitate from the clear supernatant fluid. These experiments indicate that whereas the complex formed by recombination of the 'Shiga' polysaccharide and the conjugated protein is similar in constitution and physical properties to the antigenic substance obtained by extraction of the micro-organism with diethyleneglycol, the complex formed by the polysaccharide with other proteins cannot be regarded as a true reversal of the dissociation of the 'Shiga' antigen in alkaline solution. It is to be noted that the production of the latter type of complex indicates the danger of placing too much reliance on the use of the usual acid protein precipitants to indicate the absence of protein from material containing a large percentage of undegraded bacterial polysaccharide.

Antigenicity of the artificial complexes

The complex formed by acidifying an alkaline solution of purified, undegraded polysaccharide and the 'Shiga' conjugated protein is a powerful antigen when tested in rabbits. In one experiment the complex was formed as follows: A sample (25 mg.) of the conjugated protein (11·3 % N, 0·58 % P, $[\alpha]_{5461} - 52 \pm 2°$) was dissolved in 2·5 ml. of 0·1 *N* NaOH and added to 7·5 ml. of a 1 % solution of the undegraded 'Shiga' polysaccharide prepared by the phenol method. The mixture was kept 5 min. at 25° and then cautiously acidified to about *p*H 4 by addition of acetic acid. The solution, which had a faint bluish opalescence, was evaporated to dryness *in vacuo* from the frozen state. Three doses of the artificial complex, amounting to 0·08 mg., were sufficient to produce strongly precipitating sera which also agglutinated killed suspensions of *Bact. dysenteriae* (Shiga) to about the same dilution as sera prepared against similar doses of the diethyleneglycol primary extraction product (Table 3, animals 237, 238 and 239). The samples of conjugated protein and undegraded polysaccharide used were both tested in rabbits under similar conditions and were found to be inactive (Table 3, rabbits 243, 248, 257, 260, 261 and 238, 239, 240, 241 respectively).

The same sample of polysaccharide (S 27) was combined in a similar way with the proteins, vitellin, stromatin, gelatin and serum-γ-globulin, together with the simple protein prepared by phenol dissociation of the antigenic complex and a sample of protein prepared from a 'rough' strain of *Bact. dysenteriae* (Shiga) which is described later in this paper. Two rabbits were used for each of the preparations and the immunization was continued for a total of six doses, each of 0·05 mg. In no case was any 'Shiga' precipitin or agglutinin formation

observed. Similar unsuccessful attempts to produce artificial antigens by combining the undegraded polysaccharide with such proteins as casein and denatured rabbit serum protein have already been reported [Partridge & Morgan, 1940]. On the other hand, the conjugated protein obtained from the 'Shiga' organism, and a similar substance obtained from the 'O' antigen of *Bact. typhosum* both yield polysaccharide-specific antigens on combination with a number of polysaccharides of vegetable and animal origin [Morgan & Partridge, 1940, 2; Morgan, 1941 and unpublished work]. These results, taken together, lend support to the view that in antigen formation a specific grouping in the conjugated protein is involved and that this grouping is not contained in the other protein substances examined.

The ultra-violet absorption spectrum of the amphoteric protein and the conjugated protein

The ultra-violet absorption spectra of a number of the bacterial protein preparations were determined for us by Dr R. A. Morton, to whom we express our thanks. In Fig. 1 the spectrum of a sample of the simple protein prepared by

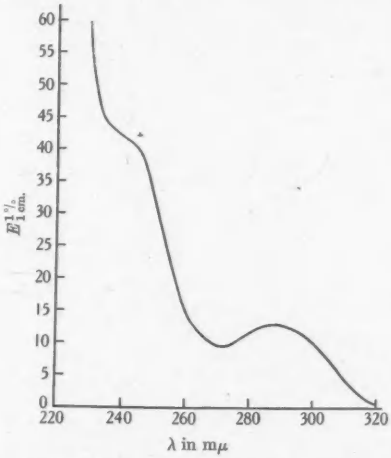

Fig. 1. Ultra-violet absorption spectrum of simple protein in 0·1 *N* NaOH.

phenol dissociation of the diethyleneglycol primary extraction product and dissolved in 0·1 *N* NaOH is recorded and is of the general type expected for substances of protein or peptide nature. The absorption at 280–290 mμ is normal for a protein containing tyrosine [Stenström & Reinhard, 1925, 1, 2], but the significance of the absorption at 235–245 mμ is not understood. Spiegel-Adolf & Seibert [1934] have shown that the pseudoglobulin in alkaline solution absorbs light selectively between 276 and 289 mμ whereas various nucleic acid preparations and their constituent pyrimidines under similar conditions absorb within the range 240–276 mμ with maximum absorption at about 265 mμ. Rough estimates of the tyrosine and tryptophan contents of the protein preparations examined have been made from the absorption data according to the method of Holiday [1936].

Using the equations given by Holiday,

$$\text{M (tyrosine)} = (1{\cdot}0\ E_{305} - 0{\cdot}092\ E_{280}) \times 10^{-3},$$

the tyrosine content of one particular sample of amphoteric protein was calculated as 7·2 %. An estimate of the tryptophan content was also derived from the equation

$$\text{M (tryptophan)} = (0{\cdot}21\ E_{280} - 0{\cdot}288\ E_{305}) \times 10^{-3},$$

and led to a tryptophan value of about 2 %. In an earlier paper the tryptophan content determined by a colorimetric method was incorrectly given as 6 %. The absorption spectrum of a sample of simple protein derived from a 'rough' variant of *Bact. dysenteriae* (Shiga) by means of phenol extraction was almost identical with that obtained for the protein substance prepared in a similar manner from a 'smooth' strain of the organism.

The ultra-violet absorption spectra of several samples of the conjugated protein were also examined and were found to be very similar to those of the simple amphoteric protein. A strong absorption at 280–290 mμ indicated the presence in the molecule of approximately the same amount of tyrosine. The intensity of the absorption within the range 230–250 mμ, however, varied considerably with different preparations of the conjugated protein, but most specimens examined gave more intense absorption within this range than preparations of the simple amphoteric protein. It has also been observed that preparations of conjugated protein contain a yellow pigment and it is not yet known whether the prosthetic group of the conjugated protein is to be identified with this chromophoric substance or with the absorption within the range 230–250 mμ. Further work on this aspect of the problem is being undertaken.

Serological comparison of the 'Shiga' proteins

It has already been demonstrated that the conjugated protein gives rise to homologous precipitins when injected into rabbits in small doses by the intravenous route [Partridge & Morgan, 1940]. Stronger sera than those previously prepared have now been obtained by giving larger doses of the protein and by limiting the operations involved in its purification. The more recent samples of conjugated protein have been prepared with the minimum of manipulation necessary to free the material from contaminating specific polysaccharide and are characterized by a higher phosphorus content. Table 5 shows the result of immunizing a group of four rabbits with a sample of conjugated protein that contained 11·3 % N, 0·58 % P and showed $[\alpha]_{5461} - 52 \pm 2°$. Further groups of rabbits were also immunized with samples of the electrophoretically pure simple protein prepared from the 'smooth' organism, and with the simple protein prepared from the 'rough' strain. When immune sera collected after the first course of antigen are employed, it appears that the anti-conjugated protein serum reacts with the homologous antigen only, whereas, after a further course of injections the strength of the conjugated protein antiserum is considerably increased and its specificity is spread so that it then reacts with the preparations of simple protein. The amphoteric proteins themselves appear to be relatively poor antigens, and the sample prepared from the 'rough' strain of *Bact. dysenteriae* (Shiga) alone engendered the formation of weak homologous precipitins. On the other hand, the antiserum to the 'rough' protein does not react with the conjugated protein or with the simple protein from the corresponding 'smooth' strain. For brevity the results obtained with pooled sera are given in Table 5, but each serum was also tested separately, and each gave similar results to those of the corresponding pooled serum samples. A sample of antitoxin prepared in

Table 5. *Precipitation reactions with proteins isolated from* Bact. dysenteriae (*Shiga*)

Serum prepared against	Immunization doses mg.	Dilutions of test antigen: Conjugated protein 1 : 10^3	Conjugated protein 1 : 10^4	Conjugated protein 1 : 10^5	Conjugated protein 1 : 10^6	Simple protein from 'smooth' strain 1 : 10^3	Simple protein from 'smooth' strain 1 : 10^4	Simple protein from 'smooth' strain 1 : 10^5	Simple protein from 'smooth' strain 1 : 10^6	Simple protein from 'rough' strain 1 : 10^3	Simple protein from 'rough' strain 1 : 10^4	Simple protein from 'rough' strain 1 : 10^5	Simple protein from 'rough' strain 1 : 10^6
Conjugated protein (pooled antisera from 4 rabbits)	1st course 3 × 0·2	2	1	1	0	0	0	0	0	0	0	0	0
	2nd course 3 × 1	3	4	3	2	2	1	0	0	3	3	1	0
	3rd course 3 × 1	4	4	3	2	2	1	0	0	4	3	2	1
Simple protein from 'smooth' strain (pooled antisera from 3 rabbits)	1st course 6 × 0·05	0	0	0	0	0	0	0	0	0	0	0	0
Simple protein from 'rough' strain (pooled antisera from 2 rabbits)	1st course 3 × 0·2	—	—	—	—	0	0	0	0	0	0	0	—
	2nd course 3 × 1	0	0	0	0	0	0	0	0	2	1	0	0
	3rd course 3 × 1	1	0	0	0	0	0	0	0	2	2	1	0

the rabbit against the toxic filtrates of the 'rough' organism [Neisser & Shiga, 1903; Conradi, 1903; Todd, 1904] gave no reaction with the test-antigens used in Table 5. A sample of toxic protein prepared by the trichloroacetic acid method of Boivin & Mesrobeanu [1937; Mesrobeanu & Boivin, 1937] was also tested against the three types of antisera given in Table 5 but no significant precipitation was observed. From these results it seems that the prosthetic group plays some role in determining the specificity of the conjugated protein.

Discussion

As a result of our earlier investigations on the chemical nature of the specific antigen of *Bact. dysenteriae* (Shiga) evidence was obtained for the view that antigenic substances prepared from *Bact. dysenteriae* (Shiga) by various methods owe their activity to the presence of two major components, a specific polysaccharide and an acidic substance that contains amino-acids and is largely polypeptide or protein in nature. Other components undoubtedly occur in the native antigen but it was found that these could be eliminated if suitable means were employed, without seriously impairing the antigenic qualities of the resulting material. The removal of these components, however, caused an alteration in the chemical and physical properties of the native antigenic complex. A method was described for the stepwise degradation of the antigenic complex which was based on the dissociating action of formamide. The non-antigenic specific polysaccharide isolated by this means could be recombined with the polypeptide-like component, obtained by acid hydrolysis of the native antigenic complex, to yield a powerful antigen that induced in rabbits the formation of immune-body specific for *Bact. dysenteriae* (Shiga).

The polypeptide-like component previously isolated from the antigenic substance by hydrolysis with dilute acetic acid at 100° contains 11·5–12·5% N and 0·6–8% P, and is readily soluble in dilute alkali but is insoluble throughout the entire acid *p*H range. An extensive amino-acid analysis of the material was not attempted, but it was shown to contain tyrosine, arginine, glutamic acid and tryptophan. The substance is digested by pepsin and by trypsin and, after acid hydrolysis, about half the total nitrogen appears as free amino-nitrogen. The low nitrogen content of the material, the poor yield of α-amino groups after acid hydrolysis and its unusual insolubility under acid conditions were considered sufficient reason for rejecting its classification as a simple protein without further investigation, and the material was at that time therefore described as a 'polypeptide-like' substance.

The results of the experiments that are described in the present paper indicate that the phospholipin-free antigenic complex can also be dissociated into its two major components by solution in 90% phenol. The addition of alcohol to the phenol solution causes the liberated polysaccharide and protein molecules to recombine and the resulting complex that is thrown out of solution is found to be antigenic. Direct dialysis of the phenol solution of the antigenic complex, however, enables the separated components to be readily isolated. The polysaccharide and protein constituents obtained by this means are unable to induce the formation of 'Shiga' agglutinins and precipitins.

In earlier attempts to purify the 'polypeptide', it was repeatedly dissolved in dilute alkali and reprecipitated by addition of acid and it was found that during this process the phosphorus content was slowly reduced while the specific rotation and nitrogen content of the substance showed only slight increases, but there was some indication that during the process the capacity of the substance to recombine with 'Shiga' polysaccharide to form an antigenic complex was

reduced, although no conclusive evidence on this point has yet been obtained. The adoption of the new technique for the dissociation of the antigenic complex in strong solutions of phenol reveals that the protein component recovered possesses markedly different properties from those of the 'polypeptide-like' substance isolated by acid hydrolysis of the native antigen. The nitrogen content approaches 14·5%, phosphorus is entirely absent and the specific rotation is considerably more *laevo*. Furthermore, the protein isolated by phenol dissociation is readily soluble in aqueous solution at *p*H 2·0–2·5 although it is insoluble at lower *p*H values than 2·0 and at higher values up to *p*H 7·0. The action of phenol on the polypeptide component itself shows that after several treatments with this solvent the bulk of the substance can be recovered as a material possessing properties very similar to, if not identical with, those of the simple protein isolated by the action of phenol on the whole antigenic complex. It becomes evident, therefore, that the polypeptide component is composed of the simple amphoteric protein and an additional substance (or substances) responsible for its acidic nature and its low nitrogen and high phosphorus contents. In the light of these observations it is suggested that the polypeptide-like component is a conjugated protein. The relationships of the different degradation products derived from the antigenic complex are set out in Fig. 2.

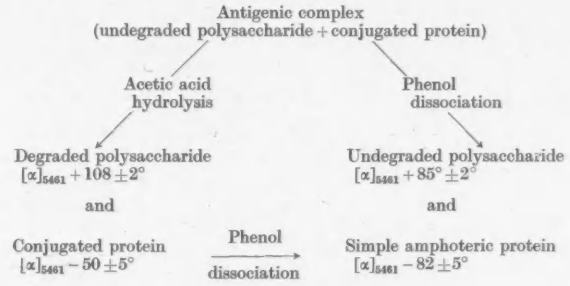

Fig. 2

It would be of considerable interest to know whether the phenol-soluble amphoteric protein which is present in both the 'rough' and 'smooth' forms of the organism is the precursor of the more complex conjugated protein component of the specific bacterial antigen and whether the development of this antigen as an additional component in the 'smooth' variant depends on enzymic mechanisms that synthesize the specific polysaccharide and convert the simple amphoteric protein into the conjugated and more reactive protein. Evidence has already been given [Partridge & Morgan, 1940] and is again demonstrated in the present paper, that under almost physiological conditions—aqueous isotonic saline solution at *p*H 8—these two components will, if present together, enter into combination and give rise to an antigenic complex. The nature of the bonds which hold the components together is unknown. It can only be stated that the forces concerned give rise to an aggregate with sufficient stability to make it behave as an independent molecular species. Furthermore, since the protein component, although essential for the manifestation of antigenicity, plays but a minor role in the determination of the immunological specificity of the antigen it would not be surprising to find a similar conjugated protein present in the specific somatic antigen of other gram-negative micro-organisms which

possess the polysaccharide-protein type of antigen. Evidence for the existence of a conjugated protein common to the antigenic complexes present in other types of gram-negative bacteria is being sought and it is of interest in this connexion to record that 'Shiga' undegraded polysaccharide combines with the conjugated protein component of the 'O' antigen of *Bact. typhosum* to form a powerful antigenic complex that induces the formation of specific 'Shiga' agglutinins and precipitins. Thus, the capacity of a specific polysaccharide of one organism to form an antigenic complex when combined with the conjugated protein component derived from the antigen of another organism that belongs to an entirely different bacterial species, has been demonstrated.

An additional method for the isolation of the polysaccharide and protein components of the native antigenic complex is also described. Antigenic material dissolved in dilute NaOH is dissociated into its two main components. If the alkaline treatment is carried out quickly and at 0° an undegraded polysaccharide is recovered on the addition of alcohol whereas the protein component can be subsequently obtained from the alcoholic supernatant fluid by the addition of acetic acid. The protein recovered appears to be identical with the simple amphoteric substance obtained by phenol treatment of the whole antigenic complex.

The polysaccharide obtained by either phenol or alkali dissociation is found to yield viscous solutions in water and to contain 0·5–1·0 % of P in organic combination. The P is not removed by the action of bone phosphatase, prolonged dialysis or by precipitation of the material from solution in ice-cold 0·05 *N* HCl by alcohol, from which it is inferred that the P is most probably an integral part of the polysaccharide molecule. Preparations of the degraded polysaccharide [Morgan, 1936] contain no appreciable quantity of P.

The viscosity values obtained for the undegraded polysaccharide exhibit a considerable variation according to the method of preparation used. It was at first thought that a determination of viscosity alone would furnish a reliable guide to the degree of degradation of the sample of polysaccharide, but it has been found that samples prepared by the phenol method occasionally have viscosity values only slightly greater than the degraded polysaccharide prepared by heating the antigenic complex in dilute acetic acid. It is to be noted, however, that phenol-treated preparations still contain 0·5–1·0 % P in organic combination and that in spite of the low viscosity, the material enters into union with the conjugated protein to yield an antigenic complex. A rapid test for ascertaining the potential capacity of polysaccharide preparations to form antigenic complexes is obviously of considerable practical value, and the most reliable guide so far found depends on the formation of soluble complexes with proteins at *p*H 4–5. On the addition of trichloroacetic acid to a mixture of conjugated protein and degraded polysaccharide a heavy precipitate of the protein immediately appears, whereas if the polysaccharide is in a form that will give rise to an antigen in combination with the conjugated protein, the solution remains clear, or, at most, develops a bluish opalescence.

It was earlier reported [Morgan & Partridge, 1940, 1; Partridge & Morgan, 1940] that the undegraded specific polysaccharide hapten of *Bact. dysenteriae* (Shiga) could be recombined with the non-antigenic conjugated protein ('polypeptide-like') component of the native antigen to form an antigenic complex that was able to induce the formation of specific 'Shiga' precipitins and agglutinins. Similarly, the conjugated protein component derived from the 'O' antigen of *Bact. typhosum* also combines with the 'Shiga' polysaccharide and forms an antigenic complex with 'Shiga' specificity. The simple protein derived

from the conjugated protein by the action of phenol, however, does not give rise to an antigenic complex in this manner and, in this respect, the simple amphoteric protein behaves like the proteins, stromatin, gelatin, human serum-γ-globulin, hen's egg vitellin and heat-denatured normal rabbit serum proteins. These observations suggest that a part of the conjugated protein molecule—most probably the prosthetic group—is necessary in order that the molecule should be capable of combining with the polysaccharide to form an antigen. The formation of a complex between undegraded specific polysaccharide and the different proteins examined certainly occurs as is shown by the behaviour of mixed solutions of the protein and polysaccharide on the addition of trichloroacetic acid. It appears unlikely that the stability of the protein under these conditions is due to the specific polysaccharide acting simply as a protective colloid, for gum acacia and other gums, which have low gold numbers [Schultz & Zsigmondy, 1903] and might be expected to function as powerful protective colloids, fail to prevent precipitation of the protein in the presence of trichloroacetic acid. In the light of these observations on the stability of proteins in the presence of an undegraded bacterial polysaccharide it is clear that the absence of a precipitate on the addition of the usual acid precipitants cannot be accepted as evidence that protein is not present in the material examined, and thus many reported observations on bacterial and tissue antigens stated to be 'protein-free' on the basis of negative tests given by trichloroacetic acid must be open to doubt. A more detailed study of these soluble and stable polysaccharide-protein complexes is being undertaken in collaboration with Dr R. A. Kekwick.

The possibility of evolving a differential extraction method whereby several organic solvents are utilized in turn for the solution and isolation of specific constituents of the bacterial cell, has already been discussed in an earlier paper [Morgan, 1937]. The results of some simple experiments along these lines are now described and it has been shown that after 6–7 % of the dry weight of the organisms employed has been removed by extraction with diethyleneglycol, it is possible by means of further extraction with strong solutions of phenol, to recover an additional 4–5 % of antigenic material together with about 10 % of a bacterial somatic protein. The antigenic material extracted from dry bacteria by 90 % phenol solution and recovered by precipitation with alcohol appears to be very similar in composition to the phospholipin-free primary extraction product obtained by means of diethyleneglycol. The free protein recovered from the bacilli, on the other hand, does not appear to be identical with the conjugated protein component of the antigenic complex. Preparations of protein which have been extracted by phenol from both 'rough' and 'smooth' cultures of *Bact. dysenteriae* (Shiga) contain 13·5–14·5 % N, are free from P and show a strongly negative rotation $[\alpha]_{5461} - 82 \pm 5°$. The protein isolated in each instance is electrophoretically homogeneous and represents at least 10 % of the dry weight of the bacterial cell.

Finally, it may be said that in common with most workers we have deemed a preparation derived from bacteria to be devoid of antigenicity when it failed to yield demonstrable homologous specific agglutinins and precipitins after it had been injected intravenously into each of a group of rabbits. Usually six doses of 0·05 mg. were given. This amount of the native antigen invariably elicits a powerful immune response with the resulting production of high titre agglutinating sera in all rabbits inoculated. For the purposes of direct comparison with other preparations from the same organism this method of measuring the antigenicity of a substance has yielded useful results, but it is necessary to emphasize that in the absence of adequate criteria of exactly what constitutes

an antigenic response it is advisable to keep in mind the results of other workers [Avery & Goebel, 1933; Chow, 1936; and Topley, 1937] who claim to have demonstrated the presence of protective immune-body in the serum of animals after immunization while being unable to detect the presence of the corresponding agglutinins or precipitins. In the absence of the latter immune-bodies the difficulty of deciding whether a preparation retains a part of the original antigenicity is still further increased when the material under test has been isolated from an organism such as Shiga's bacillus, for which the usual active and passive immunity tests that employ infection of the test animal with the living bacterium cannot readily be performed. Furthermore, it is only when adequate methods of assay exist that it is possible to detect certain changes in the qualitative nature of the immune-bodies formed. For example, Felix & Bhatnagar [1935] found that the Vi antibody elaborated in response to immunization with formolized Vi antigen is not identical with that resulting from immunization with native antigen as it occurs in the living, virulent *Bact. typhosum*. The quantitative production of Vi agglutinins appears to be normal when either type of vaccine is employed for immunization, but when sera of equal agglutinin contents are used, the phagocytosis-promoting activity and the protective power of the immune serum produced against the formolized Vi culture are much inferior to that induced after immunization with the living organism. Similar results were obtained by Henderson & Morgan [1938] who immunized rabbits with antigenic material isolated from *Bact. typhosum*. Once again, Vi agglutinins appeared in the immune serum but the protective power of the immune body as measured in passive protection tests was found to be much inferior to that given by an equal quantity of serum of equal Vi agglutinin titre prepared by immunization with living 'rough' Vi bacilli.

Summary

1. The antigenic complex of *Bact. dysenteriae* (Shiga) is soluble in concentrated (90 %) solutions of phenol. In phenol solution the complex is largely dissociated into its protein and polysaccharide components which can then be isolated in a free state by dialysis of the phenol solution.

2. The antigenic complex is partly dissociated in aqueous alkaline solution and the components can be separated by repeated fractionation of the antigenic complex from alkaline solution by alcohol.

3. The protein-free polysaccharide component prepared by either method fails to induce the formation of specific 'Shiga' agglutinins or precipitins in the rabbit.

4. Some evidence is given for the view that the acidic protein component previously described by the term 'polypeptide-like' is a conjugated protein. It is devoid of antigenicity as measured by the production of 'Shiga' immune-body, but can be recombined in aqueous solution with undegraded 'Shiga' specific polysaccharide to form a powerful antigen.

5. The protein isolated from the antigenic complex by phenol or alkali dissociation is amphoteric and has a higher nitrogen content and optical rotation than the conjugated protein. The simple amphoteric protein can also be isolated directly from the conjugated protein component of the antigenic complex. The simple protein does not form an antigenic complex when mixed with 'Shiga' polysaccharide in aqueous solution.

6. The extraction of a 'smooth' culture of *Bact. dysenteriae* (Shiga) with 90 % phenol yields the characteristic water-soluble antigenic complex and about an

equal amount of the non-specific, water-insoluble simple protein. Similar extraction of the corresponding 'rough' variant yields no antigenic material but a considerable quantity of the simple protein.

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129. MYOHAEMOGLOBIN IN THE URINE OF AIR RAID CASUALTIES WITH CRUSHING INJURY

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Patients crushed beneath fallen masonry for several hours may develop 'shock' after release, with falling blood pressure, haemoconcentration and swelling of the affected part. With restoration of circulating blood volume by serum or plasma, the blood pressure returns to normal. Some of these patients, and some also who have shown no such stage of 'shock', pass smoky dark brown or red urine, often highly acid, and giving a positive benzidine reaction. The deposit, however, seldom shows red cells, but consists chiefly of brown pigmented casts. The patient may then either develop oliguria, with progressive azotaemia, dying within the week, or may recover with a diuresis. In either case the urine becomes progressively less pigmented and the casts less numerous.

Autopsy shows blanching and necrosis of muscle in the injured areas and, microscopically, loss of staining ability; in the kidneys there are seen tubular degeneration and pigmented casts similar to those seen in the urine.

The blanched appearance of the affected muscles at autopsy suggested the possibility that this urinary pigment might be myohaemoglobin [Bywaters & Beall, 1941]; Gilmour [1941] also emphasized this likelihood, pointing out the similarity to paralytic equine myohaemoglobinuria, as Minami [1923] had done previously.

Urine from such cases has therefore been examined. This has been possible through the kind co-operation of the following: Dr Gilmour (St Andrew's Hospital, Billericay), Dr Cruickshank, Dr Pochin and Dr Hynes (North-West Hospital and Hampstead General Hospital), Dr Joan Ross (Hill End Hospital, St Albans), Prof. Pickering (St Mary's Hospital, Paddington), Mr Patey, Mr Vaughan Hudson and Dr Hardwick (Middlesex Hospital), Mr Belsey and Dr Miles (St Thomas's Hospital), Dr Dow and Mr Riddell (St George's Hospital).

Results

A. Examination with the Hartridge reversion spectroscope showed in seven cases a spectrum corresponding with that of oxymyohaemoglobin. The α band occurred at 582 mμ; after reduction with sodium hydrosulphite there was a broad band in the green. The carboxy compounds prepared from the several urines gave α bands between 578 and 579, and the 'met' compounds gave bands ranging between 633 and 636 mμ.

We prepared for comparison a solution of human myohaemoglobin from sartorius muscle (saline perfusion to wash out the erythrocytes and subsequent extraction of the minced muscle with phosphate buffer *p*H 6·5). Reference to

[1] Beit Memorial Fellow.

Table 1 will show that the bands of the urinary pigment correspond both with the values given for myohaemoglobin in the literature and with those found for the muscle extract.

Table 1. *Position of α bands in mμ*

		Oxy compound	Carboxy compound	Met compound	References
Haemoglobin		578	572	630	
Myohaemoglobin: Ox		580	—	—	Watson [1935]
		580	577	—	Roche [1932]
		582	—	—	Shenk *et al.* [1934]
		582	—	—	Gunther [1921]
	Horse	580	577	—	Roche [1932]
		581	—	—	Watson [1935]
		581	—	—	Von Mócsy [1936]
		582	579	630	Theorell [1934]
		581·5	—	638	Mörner [1897]
	Dog	580	—	—	Kennedy & Whipple [1926]
		580	577	—	Ray & Paff [1930]
		580	577	—	Roche [1932]
		580	—	—	Watson [1935]
		581	—	—	Von Mócsy [1936]
	Other mammals excluding man	580	576–577	—	Roche [1932]
		579–580	—	—	Watson [1935]
		580	—	—	Keilin [1925]
	Pigeon	580	—	—	Keilin [1925]
	Ascaris	580	—	—	
	Man	581–582	579	636	This paper
Urinary pigment from crush injury cases		582	578–579	633–636	This paper

In three cases the supernatant urine, after centrifuging, showed the bands of met-myohaemoglobin as well as those of oxymyohaemoglobin. The deposit, dissolved in a few ml. of water with a few drops of *N* NaOH, showed haematin bands, rapidly passing into those of haemochromogen. In one of these three cases the amount of pigment was estimated quantitatively by a spectrophotometric method using the pyridine haemochromogen. The first specimen, obtained 12 hr. after admission, showed the spectrum of met-myohaemoglobin with a band at 633 mμ, and was reduced to the oxy-form with α band at 582. The haem pigment concentration of the sediment and supernatant combined was in this specimen 1·08 mg./ml. (expressed as oxyhaemoglobin). The second specimen, obtained 24 hr. after admission, contained much more deposit. The total pigment concentration was 4·91 mg./ml. A third specimen, passed 31 hr. after release, contained slightly less pigment; the remaining specimens contained only traces. Thus a total of about 3–3·5 g. of myohaemoglobin was excreted in this case.

B. *Ultra-filtration.* Since myohaemoglobin has a molecular weight of 17,500 [Svedberg & Pedersen, 1940] and haemoglobin one of 68,000, separation should be possible by filtration through suitable membranes. A 9 mμ 'Gradocol' collodion filter (Elford) was used. The first specimen from the case referred to above, after reduction with sodium hydrosulphite, afforded a brown filtrate in which the band of the ferrous pigment could again be detected at 582·8 mμ. The intensity was not so great as in the original unfiltered urine. A control urine containing oxyhaemoglobin was diluted to approximately the same pigment content as the crush injury specimen and filtered through a similar 9 mμ membrane. It afforded a colourless filtrate in which no trace of haemoglobin was detectable.

C. *Ultra-centrifuging.* Dr Kekwick (Lister Institute) kindly made ultra-centrifugal runs upon these urines. They were dialysed and concentrated by freezing and then afforded the following results.

In urine from a case of crush injury, two components were present in about equal proportions, with sedimentation constants of approximately 2·3 and 4·5 respectively, suggesting that both myohaemoglobin and haemoglobin or albumin were present. The control urine containing haemoglobin showed a single component only, with a sedimentation content of approximately 4·5. (Haemoglobin and serum albumin have sedimentation constants of 4·5, whilst Svedberg & Pedersen [1940] give the value 2·0 for myohaemoglobin.)

D. *Plasma* from such patients shows no spectroscopic bands. This is to be expected since the molecule of myohaemoglobin is a quarter of the size of that of haemoglobin and the renal threshold correspondingly lower [Camus & Pagniez, 1902; Hektoen *et al.* 1928; Carlström, 1931]. It is of particular interest that no methaemalbumin was detected spectroscopically in the plasma.

E. *Renal casts.* The pigmented tubular deposit gives a positive reaction in paraffin sections by a modified benzidine reaction. Its identification as a haem pigment has been established by spectroscopic means. The technique evolved was as follows: unstained, frozen sections were laid on the microscope slide and drained. A drop of freshly prepared mixture of pyridine containing about 2 % of hydrazine hydrate was added. The granules in the tubular lumen were seen to turn pink. When viewed through the spectroscopic eyepiece, the characteristic absorption bands of pyridine haemochromogen were now visible. This proves that the pigment is a haem pigment and its general behaviour suggests that it is haematin. It is still visible in kidney material perfused before fixation. (The reaction cannot be used, however, to differentiate between myohaemoglobin and haemoglobin.)

Discussion

After these results had been obtained, references were found to cases of crushing injury seen by German observers during the last war. Minami [1923], examining post mortem material, suggested that the pigment might be myohaemoglobin and asserted his intention, if he had the opportunity, of proving it spectroscopically, but there is no record of this having being done.

Our findings prove conclusively that myohaemoglobin is excreted in the urine of such patients. It is thus direct evidence of muscle damage, and may be present even when little clinical injury is apparent. Spectroscopic examination of urine may therefore be useful in cases where the alternative possibility of haemoglobinuria due to renal contusion or mismatched transfusion exists. There have been recorded cases, however [Husfeldt & Bjering, 1937], and we have seen one such personally [Bywaters & Graham, 1941], in which a syndrome similar to that following crushing injury developed following automobile accidents, with damage to muscle blood supply but without prolonged crush. In all such cases, where 'haemoglobinuria' is associated with muscle disease or damage, spectroscopic examination should be made.

Whether muscle pigment of itself plays any part in the pathogenesis of renal failure is doubtful. It may play (*a*) a leading role, (*b*) an adjuvant role or (*c*) no role at all.

(*a*) The problem is similar to that of haemoglobinuria following mismatched transfusion. It has been shown in man that amounts of haemoglobin up to 30 ml. of corpuscles [Sellards & Minot, 1916] or 350 mg./kg. [Fairley, 1940] may be given intravenously and excreted without ill effect. Preliminary observations of

our own lead us to the opinion that small quantities of myohaemoglobin prepared from human muscle are excreted rapidly and harmlessly through the human and rabbit kidneys after intravenous injection. The few cases recorded of spontaneous 'paralytic' myohaemoglobinuria in man are important in this respect; two of the five [Paul, 1924; Gunther, 1924] died. The other three cases recovered after multiple attacks [Meyer-Betz, 1910; Hittmair, 1925; Millikan, 1939]. It seems probable that, in these cases, factors other than myohaemoglobin are concerned in bringing about the fatal outcome, since in the mildest of the three (Millikan) there was little abnormality except pigmented urine and increased blood potassium after exercise.

(*b*) As in the similar problem of haemoglobinuria after intravascular haemolysis, it may be that associated conditions such as the rate of urinary secretion, the *p*H of the urine and the amount of tubular resorption can modify the response of the kidney to the pigment. It has been claimed, for instance, on the basis of animal experiments, that the acidity of the urine is important in changing haemoglobin filtered through the glomeruli into the less soluble acid haematin or methaemoglobin [Baker & Dodds, 1925; cf. Navasquez, 1940] which may then precipitate in the tubules. Whether or not this is so in man, with an amount of excreted haemoglobin small in comparison with that used in experimental animals, it still remains to be shown that such blockage is adequate in man to produce renal failure. Experiments in this direction are in progress.

(*c*) The third possibility is that myohaemoglobin in the urine is no more than an indicator of muscle damage and that other substances released from muscle at the same time are responsible for the renal damage. The high acidity of the urine and the lowered blood alkali reserve [Bywaters & Beall, 1941] witness a considerable uptake of acid substances; it has been shown in paralytic equine myohaemoglobinuria [Grzycki, 1934] that muscle contains less phosphagen, and blood more lactic acid and phosphate, than normal. MacKay & Oliver [1935], Duguid [1936] and McFarlane [1941] have shown that phosphate in large dosage produces renal lesions in animals somewhat similar in distribution to those seen in the crush syndrome.

The study of these other substances released from muscle at the same time as pigment should not be overlooked and investigations are in progress along these lines.

Summary

Spectroscopic examination of the urine from seven patients crushed under debris showed the presence of myohaemoglobin. This was confirmed by ultrafiltration and by ultracentrifuging.

Kidney sections examined by a histochemical technique showed the presence of haem pigment within the tubules. In one case the amount of pigment excreted was measured quantitatively. The factors involved in this type of renal failure are discussed.

We are grateful to Dr Kekwick of the Lister Institute for his kind co-operation.

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130. STUDIES IN DETOXICATION

II. THE IDENTIFICATION OF CATECHOL-4-SULPHONAMIDE AS A METABOLIC PRODUCT OF *p*-HYDROXYBENZENESULPHONAMIDE IN THE RABBIT. THE SYNTHESIS OF DERIVATIVES OF CATECHOLSULPHONAMIDE

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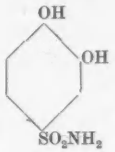
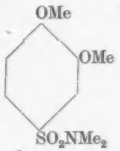
In Part 10 of this series [Sammons *et al.* 1941] it was stated that when *p*-hydroxybenzenesulphonamide (I) was fed to rabbits a small portion was oxidized to a catechol derivative, probably catechol-4-sulphonamide (1:2-dihydroxybenzene-4-sulphonamide, II). Several unsuccessful attempts had been made to isolate this compound. The reason for these failures became evident when attempts were made to synthesize it (see below) since it was found that synthetic catecholsulphonamide was a non-crystalline resinous substance. Furthermore, much difficulty was experienced in separating it from phenolsulphonamide in the urine extracts. Eventually it was found that the separation could be effected by acetylation, for whilst phenolsulphonamide was readily acetylated in aqueous solution with acetic anhydride and sodium carbonate in the cold, catecholsulphonamide was only partially acetylated. The acetylated phenolsulphonamide was insoluble and hence the main bulk of it could be removed leaving the partially acetylated catechol compound in the solution, from which it could be extracted with ether.

Since catecholsulphonamide was a resin, a search had to be made for a crystalline derivative whereby it could be characterized. Methylation of the synthetic resinous catecholsulphonamide or veratrole-4-sulphonamide with methyl sulphate and alkali gave crystalline *veratrole-4-sulphondimethylamide* (III) and this substance could be used for characterizing catecholsulphonamide. Veratrolesulphondimethylamide was obtained on suitable treatment of ether extracts of hydrolysed urine from phenolsulphonamide-fed rabbits, thus proving the presence of catechol-4-sulphonamide in the urine.

I



II



III

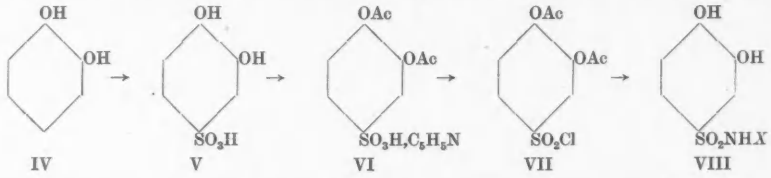
In Part 10 it was shown that about 80 % of a 2 g. dose of phenolsulphonamide could be accounted for by the ethereal sulphate and glucuronide excreted. The yield of pure veratrolesulphondimethylamide was 250 mg. and, since 24 g. of

phenolsulphonamide were fed, about 1 % of the latter was oxidized to the catechol compound. Large losses, however, are inevitable in such experiments and it is thought that much more than 1 % is oxidized; a figure of 4–5 % would probably be more correct.

This work on phenolsulphonamide was carried out in order to gain experience for tackling the problem of the oxidation products of sulphanilamide, since in Part 8 of this series [Thorpe *et al.* 1941] some evidence was obtained that, in the rabbit at least, sulphanilamide undergoes a small amount of oxidation to an *o*-aminophenolsulphonamide in a manner similar to the oxidation of phenolsulphonamide to catecholsulphonamide.

The synthesis of catecholsulphonamide derivatives

The main initial difficulty in this synthesis was the preparation of catechol-4-sulphonic acid in good yield. Several attempts were made to use the directions for the preparation of this acid given in a German patent [cf. Friedländer, 1917] which consisted of heating sodium 1:2-dichlorobenzene-4-sulphonate with NaOH in the presence of copper in a sealed tube at 220°. The results, however, were unsatisfactory. An unsuccessful attempt was also made to use the fact discovered by Paul [1906, 2] that catecholsulphonic acid was produced when guaiacol-4- and -5-sulphonic acids (both obtained during sulphonation of guaiacol at 100°) are heated to 125°. A very satisfactory method was eventually obtained in which catechol was sulphonated directly at 0°. Cousin [1893] had shown that catechol in contact with pure sulphuric acid at 0° for several days was converted into a monosulphonic acid. Later, Gentsch [1910] showed that this acid was catechol-4-sulphonic acid and not the -3-sulphonic acid as had been previously thought, since on methylation it gave veratrole-4-sulphonic acid, a compound of known structure [Paul, 1906, 1]. The sulphonic acid (V) obtained in this way was acetylated with acetic anhydride to give the *pyridine salt* (VI)



IV V VI VII VIII

which was easily converted into *1:2-diacetoxybenzene-4-sulphonyl chloride* (VII). From this chloride, sulphonamides of the type represented by VIII (X = H or other group) could be made.

Experimental

Identification of catecholsulphonamide in urine after phenolsulphonamide. Phenolsulphonamide (2 g.) dissolved in warm water was administered (stomach tube) daily for 3 days to each of four rabbits. The urine was collected daily for 4 days following the first dose of the sulphonamide (see Table 1). Each batch of urine was hydrolysed by boiling for an hour with 1/5 vol. of conc. HCl. The hydrolysed urine was filtered and then extracted with ether in a large continuous extractor of 1 l. capacity. The ethereal extracts were combined and dried for a short time with anhydrous calcium chloride. After filtration, the solution was evaporated under diminished pressure leaving a blackish residue consisting of tarry material mixed with crystals. The residue was now dissolved in 400 ml. water and filtered to remove some black insoluble matter. The clear brownish

Table 1. *Extraction of sulphonamides from urine*

Day	No. of rabbits	Total phenol-sulphonamide fed g.	Urine vol. ml.	Extraction time hr.
1	4	8	—	—
2	4	8	650	8
3	4	8	800	20
4	4	0	580	20
5	4	0	500	20
		Total 24		

filtrate, which gave an intense green colour with $FeCl_3$, was treated with 20 g. anhydrous Na_2CO_3 and when the latter had dissolved, 16 ml. of acetic anhydride were added. The whole was thoroughly mixed by shaking and after a short time acetoxybenzenesulphonamide separated. After 1 hr. at 0°, the acetyl compound was filtered off and washed well with water. The combined filtrates (*ca.* 700 ml.) were slightly acid in reaction and gave a faint catechol $FeCl_3$ reaction. This reaction became very intense after heating the solution with a little dilute NaOH. The acetyl compound (13·4 g.) gave only a faint colour with $FeCl_3$ under these conditions, showing that nearly all the catechol compound was in the filtrates. The latter was now evaporated *in vacuo* at 50° and during this process a further 0·7 g. of acetoxybenzenesulphonamide was recovered. The residue from this evaporation was semi-solid and bulky and contained much sodium acetate derived from the acetylating reagents. It was therefore dissolved in 200 ml. water, made strongly acid with HCl and then extracted for 16 hr. with ether in a continuous extractor. The ethereal extract was evaporated leaving a clear brownish syrup, which gave an intense green colour with $FeCl_3$ and reduced ammoniacal $AgNO_3$ in the cold.

Methylation of the syrup and isolation of veratrolesulphondimethylamide. The whole of the above syrup, after a preliminary trial on a small sample, was methylated by stirring with 12 ml. methyl sulphate and 30 ml. of 10 % NaOH, each added in three portions during 1 hr. at room temperature. A little acetone was also added during the methylation. The solution was then heated on the water bath for 20 min. to remove acetone and destroy excess methyl sulphate, the solution being kept alkaline by small additions of NaOH. During the heating an oil separated and, on keeping the solution at 0°, crystals separated and the oil solidified. The solid (1 g.) was filtered off, dried and then recrystallized several times from hot water. The purified crystals (250 mg.) were identical with authentic veratrolesulphondimethylamide and formed long rectangular leaflets. (Found: C, 49·0; H, 6·3; S, 12·8 %. $C_{10}H_{15}O_4NS$ requires C, 49·0; H, 6·2; S, 13·1 %.) They melted at 112°,[1] not depressed by the authentic substance, but depressed considerably by authentic anisolesulphondimethylamide. Both the above sample and the synthetic sample of veratrolesulphondimethylamide appear to sinter at about 105° unless very carefully dried before taking the melting point.

Synthetic experiments

Veratrole-4-sulphondimethylamide. Veratrole was converted successively into potassium veratrole-4-sulphonate, veratrole-4-sulphonyl chloride and -4-sulphonamide according to Paul [1906, 1]. The sulphonamide (1 g.) was dissolved in 30 ml. 10 % NaOH and methylated with 8 ml. methyl sulphate with vigorous stirring. After about 10 min. the solution became warm and a white solid began to separate. Stirring was continued for another 30 min. and then the alkaline

[1] Melting points are uncorrected.

solution was diluted with water. The white solid was filtered off, thoroughly washed with water and recrystallized from a large volume of hot water. The yield of *veratrole-4-sulphondimethylamide* was almost quantitative. It formed long rectangular leaflets, insoluble in alkali and cold water, sparingly soluble in hot water and soluble in alcohol; M.P. 115°. (Found: C, 49·6; H, 6·4; N, 5·3 %. $C_{10}H_{15}O_4NS$ requires C, 49·0; H, 6·2; N, 5·7 %.)

*Anisole-*p*-sulphondimethylamide.* Phenol-*p*-sulphonamide (1 g.) was shaken with 8 ml. methyl sulphate and 20 ml. 15 % NaOH, a little methyl alcohol being added to facilitate mixing. The mixture was shaken for 15 min. and then heated on a water bath for about 15 min. During this time an oil separated which solidified on cooling. The solid *anisolesulphondimethylamide* was recrystallized from a large volume of hot water. It formed plates, M.P. 75°. (Found: C, 50·4; H, 5·9 %. $C_9H_{13}O_3NS$ requires C, 50·2; H, 6·1 %.)

1:2-Diacetoxybenzene-4-sulphonyl chloride. 5 g. of well-powdered catechol were added to 6 ml. conc. H_2SO_4. The catechol quickly dissolved and the viscous solution was kept at 0° for a week. By this time the mixture had become an almost solid crystalline mass. The latter was broken up and dissolved in about 300 ml. water and the excess of H_2SO_4 removed with $BaCO_3$. The solution was boiled and filtered from $BaSO_4$. Any barium left in the filtrate was precipitated by adding the requisite amount of 5*N* H_2SO_4 and filtering. The filtrate, containing the catecholsulphonic acid, was evaporated to a syrup *in vacuo* at 50–60°; the syrup was then dried. No attempt was made to crystallize the acid (Cousin [1893] states that on evaporating a solution in a desiccator, catecholsulphonic acid forms crystals, M.P. 53–54°). The dry syrup was dissolved in 12 ml. pyridine with cooling under the tap and the solution treated with 12 ml. acetic anhydride, again with cooling. The mixture was kept overnight at room temperature. The resulting thick liquid was concentrated *in vacuo* at 50° to a stiff syrup which did not crystallize. This product is, presumably, *pyridine 1:2-diacetoxybenzene-4-sulphonate*; it was easily soluble in water and its solution gave no coloration with $FeCl_3$. The syrupy pyridine salt was now triturated with 12 g. powdered PCl_5 added in small portions. There was a vigorous reaction with much heat. The resulting mobile yellow liquid was poured into 1 l. cold water and stirred until it solidified. The solid was broken up and kept several hours in the water. The water was now changed and, after finely powdering, the whole was kept overnight. The *1:2-diacetoxybenzene-4-sulphonyl chloride* was filtered off and dried (yield, crude, 10·3 g.). It was recrystallized from carbon tetrachloride and formed large elongated hexagonal plates, M.P. 116°. (Found: C, 41·1; H, 2·9; S, 10·65; Cl. 12·1 %. $C_{10}H_9O_6SCl$ requires C, 41·0; H, 3·1; S, 10·95; Cl, 12·1 %.) On keeping even the pure chloride for about 2–3 months, it decomposed leaving a syrupy fuming mass.

Catechol-4-sulphonamide. 5 g. of the foregoing chloride were stirred into 20 ml. of a solution of equal parts water and ammonia solution (sp. gr. 0·880). The chloride gradually dissolved and the solution was kept overnight. The solution was now acidified with 3*N* HCl, thereby becoming cloudy, and oily globules separated on standing. The solution was extracted with ether in a continuous extractor for 12 hr. Evaporation of the ether left a clear brown resin (2 g.) which could not be crystallized. Its aqueous solution gave, with ferric chloride, an intense green colour turning blue-green, purple and finally red on adding dilute Na_2CO_3, and it reduced ammoniacal $AgNO_3$ in the cold. The resin was *catechol-4-sulphonamide*, since on methylation with methyl sulphate and alkali it gave veratrole-4-sulphondimethylamide, M.P. 113° not depressed by an authentic sample.

1:2-Diacetoxybenzene-4-sulphonanilide. A saturated solution of the above chloride (1 mol.) in purified ethyl acetate was mixed with a saturated solution of aniline (2 mol.) in the same solvent [cf. Fosbinder & Walter, 1939]. The mixture was kept at room temperature overnight. During this time the hydrochloride of the base crystallized. This was filtered off and the filtrate evaporated on the water bath. The residual oil solidified on stirring with water and the solid was recrystallized from aqueous alcohol. The yield was almost quantitative. The *sulphonanilide* formed elongated hexagonal plates, M.P. 127–128°. (Found: S, 9·0 %. $C_{16}H_{15}O_6NS$ requires S, 9·2 %.) *1:2-Diacetoxybenzene-4-sulphon*-m-*chloranilide* was prepared in a similar manner from *m*-chloroaniline. It formed needles from aqueous alcohol, M.P. 153°. (Found: S, 8·3; Cl, 9·4 %. $C_{16}H_{14}O_6NSCl$ requires S, 8·35; Cl. 9·2 %.)

1:2-Diacetoxybenzene-4-sulphon-β-naphthylamide was similarly prepared from β-naphthylamine. It formed long plates from aqueous methyl alcohol, M.P. 131°. (Found: S, 8·05 %. $C_{20}H_{17}O_6NS$ requires S, 8·0 %.)

Catechol-4-sulphonanilide. Diacetoxybenzenesulphonanilide was dissolved in cold 5 % NaOH. After 5–10 min. the solution was acidified with dilute HCl and the *catecholsulphonanilide* separated on standing. The crystals were filtered off, dried and recrystallized from carbon tetrachloride (yield almost quantitative). It formed white glistening plates, M.P. 225° (decomp.), very sparingly soluble in water and had no action on a 1 in 50 blood solution at room temperature during 24 hr. (Found: C, 54·1; H, 4·4; N, 5·3 %. $C_{12}H_{11}O_4NS$ requires C, 54·3; H, 4·2; N, 5·3 %.)

Catechol-4-sulphon-m-*chloranilide* was similarly prepared and formed plates from hot water, M.P. 177°. (Found: C, 48·25; H, 3·7; N, 4·7 %. $C_{12}H_{10}O_4NSCl$ requires C, 48·1; H, 3·4; N, 4·7 %.)

Catechol-4-sulphon-β-naphthylamide was similarly prepared and formed plates from water with a little alcohol, M.P. 218° (decomp.). (Found: C, 61·3; H, 4·2; N, 4·5 %. $C_{16}H_{13}O_4NS$ requires C, 60·9; H, 4·1; N, 4·4 %.) The above catechol derivatives were all very sparingly soluble in cold water, but the solutions gave a green colour with $FeCl_3$ and reduced ammoniacal silver nitrate in the cold. They did not reduce Fehling's solution.

Summary

It has been shown that when phenol-*p*-sulphonamide is fed to rabbits, a small portion is oxidized to catechol-4-sulphonamide. The latter was isolated from acid-hydrolysed urines and characterized as the crystalline methylated derivative, veratrole-4-sulphondimethylamide, a synthetic sample of which had been made from veratrole-4-sulphonamide, a compound of known structure.

About 1 % of the phenolsulphonamide fed was actually accounted for, as pure veratrolesulphondimethylamide, but it is probable that the actual amount oxidized is about 4–5 %.

The synthesis of catechol-4-sulphonamide is described. It was found to be a non-crystalline, resinous substance. Crystalline derivatives such as catechol-sulphonanilide etc. have also been described.

The elementary analyses quoted in this paper were carried out by the micro-analyst at the Chemistry Department, Birmingham, and the author wishes to thank Dr M. Stacey for making the necessary arrangements.

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131. STUDIES IN DETOXICATION

12. THE METABOLISM OF VANILLIN AND VANILLIC ACID IN THE RABBIT. THE IDENTIFICATION OF GLUCUROVANILLIN AND THE STRUCTURE OF GLUCUROVANILLIC ACID

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WHEN aldehydes are fed to animals, the —CHO group is either reduced to —CH_2OH or oxidized to —COOH. It can be stated broadly that those aldehydes undergoing biological reduction are aliphatic in nature, whilst those oxidized to the corresponding acid are aromatic.[2] Examples of the former are chloral [Musculus & Mering, 1875; Mering, 1882], butyl chloral [Mering, 1882] and bromal [Maraldi, 1903]. Besides these halogenated derivatives few other aliphatic aldehydes have been studied although Neubauer [1901] states that he could not detect conjugated glucuronic acid in the urine of dogs and rabbits after feeding *iso*butaldehyde, *iso*valeraldehyde and heptaldehyde; Cohn [1893] was also unable to detect any metabolic products after acetaldehyde and paraldehyde. These aldehydes probably undergo complete oxidation *in vivo*, since Boyland [1940] has recently stated that heptaldehyde appears to be completely oxidized in the rabbit, as he was unable to detect any of its possible metabolic products (heptyl alcohol, heptoic and pimelic acids) in the urine. Aromatic aldehydes without exception are oxidized to the corresponding acids, e.g. benzaldehyde, substituted benzaldehydes, thiophene aldehyde [Cohn, 1893] and *p*-nitrophenylacetaldehyde [Sherwin & Hynes, 1921]. Furfuraldehyde also behaves as an aromatic aldehyde, but, unlike the others, it forms in dogs and rabbits (but not in hens) small amounts of furfuracrylic acid which is excreted in conjugation with glycine [Jaffe & Cohn, 1887; 1888; Cohn, 1893]. The open chain terpene aldehyde, citral (geranial) has its —CHO group oxidized to —COOH *in vivo* [Kuhn *et al.* 1936], but the closely related aldehyde, citronellal, deserves special mention since it is cyclized in the rabbit, forming a menthoglycol, and it appears that ring formation takes place in the stomach under the influence of the gastric HCl [Kuhn & Löw, 1938].

The metabolism of vanillin

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is a typical aromatic aldehyde as regards its fate in the animal body. It is widely used in the perfumery and confectionery trades and is considered to be responsible for a skin condition

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[2] It is interesting to note in this connexion that there are found in animal tissues two enzymes acting on aldehydes, namely aldehyde mutase and aldehyde oxidase (Schardinger enzyme). The former appears to have no action on aromatic aldehydes but causes the dismutation of aliphatic aldehydes, the —CHO group forming both a —CH_2OH and a —COOH. The latter acts on both aliphatic and aromatic aldehydes and converts the —CHO group into —COOH [see Dixon, 1939]

sometimes found in those handling it; it may also be the cause of vanillaism, a dermatosis affecting persons handling vanilla pods [see Schwartz & Tulipan, 1939; White, 1934]. The fate of vanillin in the animal body was first studied by Preusse [1880] who fed both vanillin and an alcoholic extract of vanilla pods to rabbits. He found both to increase the ethereal sulphate output and form vanillic acid which was mainly excreted free but some as an ethereal sulphate. Preusse also stated that a very small amount of vanillin was excreted unchanged. Cohn [1893] confirmed Preusse in showing that vanillin was oxidized to vanillic acid *in vivo* and later Marfori [1897] showed that *iso*vanillin, methylvanillin (veratraldehyde) and protocatechuic aldehyde were similarly oxidized to the corresponding acids. Whilst Preusse [1880] also demonstrated that urine after vanillin administration contained a laevorotatory substance, it was left to Kotake [1905] to isolate it (as a Ba salt) and show it to be the conjugated glucuronide of vanillic acid which he called glucurovanillic acid (cf. the glucoside, glucovanillic acid). As far as we are aware, no previous study of the fate of vanillic acid, as distinct from vanillin, has been carried out.

The results of the present work, which is an attempt to give a quantitative picture of the fate of vanillin and vanillic acid in the rabbit, are summarized in Fig. 1 (see also Tables 3 and 4). About 83 % of the vanillin fed (dose 1 g./kg.)

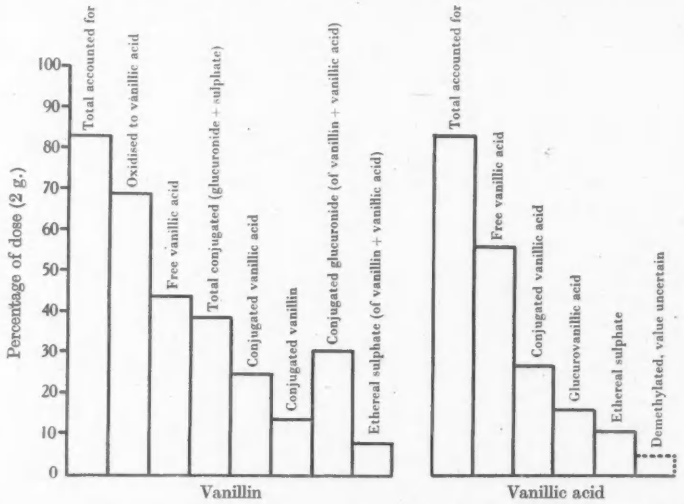


Fig. 1. Diagram illustrating quantitatively the distribution of metabolic products in the urine of rabbits after a 2 g. dose of vanillin or vanillic acid.

has been accounted for; 69 % was oxidized to vanillic acid, 44 % being free and 25 % being conjugated vanillic acid. The latter includes both ethereal sulphate and conjugated glucuronide. Some 8 % of the vanillin was excreted as an ethereal sulphate. The other 14 % of the vanillin accounted for was excreted conjugated but not oxidized, the total vanillin and vanillic acid excreted in conjugation being 39 %. Two conjugated glucuronides were excreted, namely glucurovanillin (*p*-aldehydo-*o*-methoxyphenylglucuronide) and glucurovanillic acid (*p*-carboxy-*o*-methoxyphenylglucuronide). The former was isolated as the *β-naphthyl-*

hydrazone, M.P. 179°, and the latter characterized as the *dimethyl ester* of *trimethylglucurovanillic acid*, M.P. 137°. Demethylation of vanillin was not detected (cf. vanillic acid) nor was any other form of detoxication, such as hippuric acid formation, found.

The excretion of glucurovanillin

In previous work on the fate of vanillin [Preusse, 1880; Cohn, 1893; Kotake, 1905], the only metabolic products detected were free and conjugated vanillic acid. Preusse stated that a small amount of vanillin was excreted unchanged, but his evidence—the smell of vanillin—was inconclusive. In the present work, conjugated but not unchanged vanillin was found. The conjugated vanillin in which the aldehyde group survives unchanged, was only found in urines collected up to 5–6 hr. after the administration of vanillin; glucurovanillic acid was also being excreted during this time. The presence of the conjugated vanillin in the urine could be detected within an hour of feeding, even after comparatively small doses of vanillin (200 mg./kg.). After the first 5–6 hr., conjugated glucuronide was still being excreted, presumably glucurovanillic acid, but the excretion of conjugated vanillin ceased fairly abruptly (see Table 1).

On keeping urine containing conjugated vanillin, the amount of the latter diminished and fell to a small value after a week. Boiling the urine retarded the diminution slightly (see Table 4). This disappearance of conjugated vanillin on standing in urine is either due to bacterial action or to aerial oxidation, but not to

Table 1. *The excretion of conjugated vanillin*

Time after administration of vanillin (2 g.) hr.	Rabbit no. 71	72	74	75	76
0–1	n.u.	n.u.	+	n.u.	+
1–2	+	+	+	+	+
2–4	n.u.	n.u.	+	+	+
4–6	+	+	±	n.u.	±
6 7	–	n.u.	–	±	–
7–24	–	–	–	–	–

+ urine gave red colour with naphthoresorcinol and HCl in the cold, i.e. presence of conjugated vanillin.
± red colour very weak.
– absence of conjugated vanillin.
n.u. no urine excreted during that period.
All samples of urine collected during the 24 hr. test period gave strong positive reactions for glucuronic acid, the earlier ones giving the strongest tests.

any oxidizing enzyme being excreted simultaneously with the conjugated vanillin. We hope to investigate this point with vanillin and other aldehydes some time in the future. These observations were extended to other aromatic aldehydes, and it was found that those containing an OH group *para* to the —CHO formed, in the rabbit, conjugated aldehydes which were excreted in the first 5–6 hr. after feeding. If the OH group was methylated, then none of the aldehyde group survived and an ester glucuronide was excreted thus giving urines which reduced Benedict's and Fehling's solutions (see Table 2). It appears therefore, that a benzaldehyde possessing a *p*-OH group forms in the rabbit a conjugated aldehyde of the type $p\text{-}R\text{O}.\text{C}_6\text{H}_4.\text{CHO}$ and a conjugated acid, $p\text{-}R\text{O}.\text{C}_6\text{H}_4.\text{COOH}$, whilst a benzaldehyde possessing a *p*-OMe forms an ester glucuronide of the type $p\text{-MeO}.\text{C}_6\text{H}_4.\text{COO}R$ (*R* represents glucuronic acid). We have already isolated the conjugated glucuronides of vanillin, *p*-hydroxybenzaldehyde and protocatechuic aldehyde as 2:4-dinitrophenylhydrazones. The

ester glucuronides, veratroyl and anisoyl glucuronides, formed after feeding veratraldehyde and anisaldehyde respectively, have also been isolated: the ester glucuronides after piperonal and *o*-methoxybenzaldehyde have not yet been isolated although their presence has been detected. The main product of piperonal metabolism was piperonyloylglycine ('piperonylursäure' of Heffter [1895]). The preparation and properties of these ester glucuronides will be the subject of a future communication.

Table 2. *Excretion of conjugated aldehydes and ester glucuronides*

Aldehydes* forming conjugated aldehydes and acids in the rabbit	Aldehydes† forming ester glucuronides in the rabbit
p-Hydroxybenzaldehyde	Anisaldehyde
Vanillin	Veratraldehyde
Protocatechuic aldehyde	Piperonal
2:4-Dihydroxybenzaldehyde	*o*-Methoxybenzaldehyde

* Urines after these aldehydes gave the 'cold N.R. test' (see later), the Tollens glucuronic acid test, and were non-reducing.

† In these cases, the urines were strongly reducing, gave the Tollens test, but not the 'cold N.R. test'.

The detection of conjugated vanillin was made by means of a colour reaction which is described in the appendix to this paper. *p*-Hydroxy- and *p*-methoxybenzaldehydes were found to give an intense red colour with naphthoresorcinol and strong HCl in the cold, whilst the corresponding acids gave no colour under the same conditions. Vanillin, since it contains the *p*-hydroxybenzaldehyde grouping, also gave the test, and since, under the conditions of the test, i.e. using strong HCl, conjugated vanillin is hydrolysed, then urines containing it (as glucuronide or ethereal sulphate) give a deep red colour with naphthoresorcinol and HCl in the cold. This test will be referred to as the 'cold N.R. test' in this paper, but it must not be confused with the Tollens test for glucuronic acid which also uses naphthoresorcinol and HCl but requires heating.

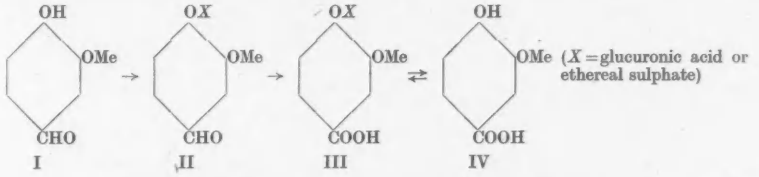
The metabolism of vanillic acid

As in the case of vanillin, some 83 % of the vanillic acid fed (1 g./kg.) could be accounted for (see Fig. 1 and Table 3), 56 % being excreted free and 27 % conjugated. The conjugated vanillic acid includes ethereal sulphate (11·3 %) and glucuronide (15·8 %). At present there appears to be no simple way of deciding whether the vanillic acid which is excreted in the free state has passed through the organism unconjugated, although it is possible that there may be an equilibrium between the conjugated and unconjugated forms. Unlike vanillin, vanillic acid undergoes some demethylation in the rabbit. We were not able to determine the extent of this demethylation, but consider it unlikely to be more than 5 % of the vanillic acid fed. Other work in this laboratory has shown also that veratric aldehyde (3:4-dimethoxybenzaldehyde) undergoes a double demethylation in the rabbit. Examples of demethylation of aromatic methoxyl groups have been given recently by Stroud [1940], namely, the cases of 4-methoxydiphenyl ether and 4:4′-dimethoxydiphenyl ether in the rabbit. The demethylation of vanillic acid may account for the slightly higher sulphate conjugation (11·3 %) of this acid compared with vanillin (8·1 %), since the product of the demethylation, protocatechuic acid, has two phenolic hydroxyls which may both conjugate with sulphate. Some of this protocatechuic acid is excreted in the free state, since

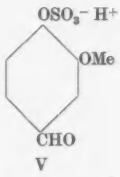
urine, after feeding vanillic acid, gave an intense green colour with $FeCl_3$ before hydrolysis. Preusse [1878–9] has shown that protocatechuic acid when fed to dogs is excreted partly unchanged, partly conjugated and partly decarboxylated to catechol. The possibility that vanillic acid might be decarboxylated had therefore to be considered, but we were unable to detect guaiacol in urine after vanillic acid.

The mechanism of the detoxication of vanillin and vanillic acid

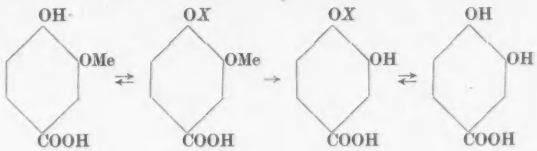
The fact that some of the vanillin (I) fed appears in the urine as conjugated vanillin (II), and this only during the few hours immediately after feeding, may indicate that the first stage in the metabolism of vanillin is conjugation of the OH group and this appears to be a fairly quick reaction. The next stage would be the oxidation of the —CHO group with the formation of conjugated vanillic acid (III). The latter would, presumably, then be in equilibrium with free vanillic acid (IV) formed by hydrolysis of the conjugated form or possibly by direct oxidation of some of the vanillin:



The stage III–IV is written as being reversible since both free and conjugated vanillic acid are excreted after feeding vanillic acid. It may be that conjugation of the phenolic hydroxyl is a necessary antecedent to the biological oxidation of the aldehyde group. On theoretical grounds, the aldehyde group of vanillin would be more easily oxidized if a group which would tend to create a drift of electrons towards the —CHO group were substituted for the OH. The ethereal sulphate (V) or the glucuronide could act as such a group because they are strongly acidic substances and on ionization would leave a negative charge *para* to the —CHO group:



In the case of vanillic acid, the group in the *p*-position to the hydroxyl (i.e. the COOH) is already oxidized, so that oxidation now takes place in the *o*-position to the OH resulting in demethylation thus:



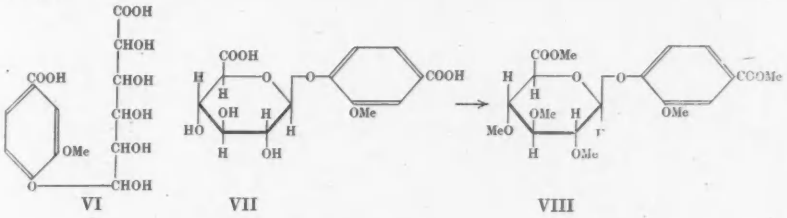
Here again it is possible that conjugation of the OH would make the demethylation easier. It appears, however, that the demethylation must be a slow process, since in the case of vanillin it was not detected; this may be because of the extra steps involved in the preliminary formation of vanillic acid which would therefore not stay long enough in the body to suffer further oxidation. It should be noted in this connexion that during oxidations involving the benzene ring in the rabbit, *p*-oxidation rather than *o*-oxidation is the predominant process. *Ortho*-oxidation seems more characteristic of the dog and there are several examples to be found in the literature illustrating this species difference [see papers of Horn, 1936, 1, 2; 1937].

The structure of glucurovanillic acid

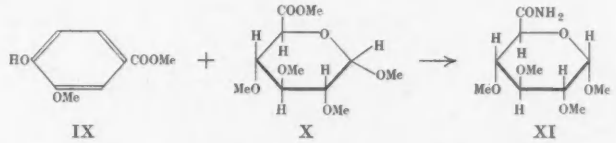
Kotake [1905] showed that the conjugated glucuronide present in rabbit urine following the administration of vanillin was composed of vanillic and glucuronic acids. The vanillic acid was combined with glucuronic acid through its phenolic group; the acid was dibasic since one molecule of the glucuronide was combined with one atom of Ba in the Ba salt. Hildebrandt [1906] showed that potassium glucurovanillate was hydrolysed by emulsin and was therefore a β-glycoside.

Analysis of the Ba salt [Kotake, 1905] necessitated the formula $BaC_{14}H_{16}O_{11}$ which contains a molecule of water more than is required for the barium salt of a glucuronide of vanillic acid, $BaC_{14}H_{14}O_{10}$. This molecule of water was not removed by drying over P_2O_5 at 100° and therefore Kotake concluded that it was attached to the aldehyde group of the glucuronic acid. On the basis of these facts the structure of glucurovanillic acid was given by Kotake as VI. This substance on hydrolysis was supposed to give vanillic and glucuronic acids without the addition of water. In our experience, the Ba salts of phenolic glucuronides are very difficult to purify, since they are usually amorphous powders and can only be purified by the unsatisfactory method of precipitation from aqueous solution by alcohol. A structure based on the elementary analysis of such a salt cannot be considered to be much more than a guess. We failed completely to obtain a pure Ba salt, but by methylation of the crude salt, we were able to characterize the acid and determine its structure. In the present work we have obtained definite evidence that glucurovanillic acid has the structure represented by VII. Methylation of the crude Ba salt gave the crystalline *methyl ester of 2:3:4-trimethyl*-o-*methoxy*-p-*carbomethoxyphenyl*-β-d-*glucuronide* (trimethylglucurovanillic acid dimethyl ester), VIII, M.P. 137° and $[\alpha]_D^{23°}$ −86·05° (in $CHCl_3$). The crude Ba salt contained, in addition to the glucurovanillate, Ba vanillate, the latter being isolated from the product of methylation as veratric acid and its methyl ester. Hydrolysis of the fully methylated glucurovanillic acid with methyl alcoholic HCl gave a syrupy mixture ($[\alpha]_D^{20°}$ +48·2° in $CHCl_3$) of methyl vanillate, IX, and the liquid methyl ester of 2:3:4-trimethyl-$\alpha\beta$-methylglucuronide, X. The latter substance has been shown to be a pyranoside by Pryde & Williams [1933] and Smith [1939] has shown that it can be conveniently characterized as the crystalline amide (XI) of 2:3:4-trimethyl-α-methylglucuronide. The mixture of the esters, IX and X, was purified by distillation in a high vacuum, though no appreciable separation was obtained in this way. Their separation was achieved by the fact that whilst the methylated glucuronide, X, forms an amide on treatment with methyl alcoholic ammonia, methyl vanillate does not. The isolation of the crystalline amide, XI, M.P. 182° and $[\alpha]_D$ +135° (in water) and methyl vanillate, IX,

M.P. 63°, therefore determines the structure of fully methylated glucurovanillic acid as VIII and hence glucurovanillic acid must be *o*-methoxy-*p*-carboxyphenyl-*β*-*d*-glucopyranuronoside, VII. The fact that, when fully methylated glucurovanillic acid is hydrolysed, the rotation changes from negative to positive confirms the observation of Hildebrandt [1906] that the glycosidic link is of the *β*-type.



VI VII VIII



IX X XI

It is also reasonable to infer that glucurovanillin can be represented by VII if one substitutes a —CHO group for the aromatic —COOH, since it appears that glucurovanillic acid is formed *in vivo* from glucurovanillin.

Experimental

The vanillin (B.D.H.) used in these experiments had M.P. 82°.[1] The vanillic acid used was prepared from guaiacol by refluxing the latter with carbon tetrachloride and NaOH in the presence of freshly prepared copper powder (from Zn and $CuSO_4$), a method based on a German patent [Friedländer, 1915]. The acid was purified by sublimation *in vacuo* and had M.P. 207°. (Found: OMe, 18·2%. $C_8H_8O_4$ requires OMe, 18·45%.)

Analytical methods

Both vanillin and vanillic acid were fed to rabbits by stomach tube as fine powders suspended in warm water. All rabbits used were females weighing approx. 2 kg.

Ethereal sulphate. Determination of ethereal sulphate in the urine after administration of the above substances, together with the appropriate controls, was carried out exactly as described in Part I of this series [Williams, 1938].

Free vanillic acid. The most satisfactory method found for the determination of vanillic acid was based on the fact that it possesses a methoxyl group. 10 ml. urine mixed with 1 ml. 10% H_2SO_4 were extracted for 2 hr. with ether in a small continuous extractor. The extract was carefully evaporated to dryness to remove all traces of ether and the residue was then transferred in acetone to the reaction flask of a semi-micro Zeisel methoxyl apparatus. The acetone was now carefully

[1] Melting points are uncorrected.

boiled away and a methoxyl determination carried out on the residue in the usual manner. Control experiments showed that vanillic acid added to urine in the amounts likely to be encountered in these experiments could be estimated by this method with an error of not more than 5%.

Total vanillic acid and vanillin (i.e. free + conjugated). 20 ml. urine and 4 ml. conc. HCl were gently boiled for 30 min. The mixture was cooled and made up to 25 ml. in a measuring flask. 10 ml. of this solution were now extracted continuously with ether and the procedure described above for the determination of free vanillic acid was then followed exactly. Control experiments showed that vanillic acid added to urine which was then boiled with conc. HCl could be estimated with an error of not more than 5%; vanillic acid was not demethylated under these conditions.

Table 3. *The metabolism of vanillin and vanillic acid*

Substance fed	Rabbit no.	Dose g.	Free vanillic acid		Conjugated vanillin and/or vanillic acid				Total vanillin and/or vanillic acid	
					* Total	As sulphate		* As glucuronide		
			g.	%	%	g.	%	%	g.	%
Vanillin	72	2·00	0·882	44·1	36·2	0·172	8·6	27·6	1·607	80·3
	76	2·01	0·929	46·2	37·8	0·131	6·5	31·3	1·690	84·1
	78	2·04	0·865	42·5	42·8	0·189	9·3	33·6	1·739	85·2
			Average	44·3	38·9		8·1	30·8		83·2
Vanillic acid	78	2·02	1·014	50·1	28·1	0·209	10·3	17·8	1·584	78·4
	76	2·01	1·240	61·7	21·2	0·188	9·4	12·8	1·680	83·7
	71	2·00	1·140	57·0	31·0	0·287	14·3	16·7	1·774	88·7
			Average	56·3	26·8		11·3	15·8		83·6

* The figures in these columns were obtained by difference.

Table 4. *Estimation of conjugated vanillin*

Rabbit no.	Dose g.	Urine vol. in 6 hr. ml.*	Vanillin 2:4-dinitrophenylhydrazone from 10 ml. urine mg.	Vanillin conjugated g.	% Vanillin conjugated but not oxidized	% Conjugated vanillin in urine after standing a week
63	2·0	140	41·9	0·268	13·4	1·3
65	2·0	191	30·5	0·266	13·3	1·7 (6·1 in boiled urine)
66	2·0	207	32·2	0·305	15·2	7·3
74	2·0	237	27·6	0·299	14·9	—
75	2·0	145	45·1	0·299	15·0	—
					Average 14·4	

* Urines collected after this time contained no conjugated vanillin.

Conjugated vanillin. 10 ml. urine were hydrolysed by boiling for 30 min. with 2 ml. conc. HCl. The hydrolysed urine was cooled and then extracted with ether for 2 hr. in a continuous extractor. The ether was evaporated, the residue dissolved in a little alcohol and then diluted with 20–30 ml. water. The solution was then treated with excess (20 ml.) of a 0·5% alcoholic solution of 2:4-dinitrophenylhydrazine hydrochloride. After 30 min. the vanillin 2:4-dinitrophenylhydrazone was collected on a sintered glass filter, washed with dilute HCl and finally with water. The whole was dried at 100° and the hydrazone weighed.

Isolation of glucurovanillin-β-naphthylhydrazone

Six rabbits were each fed with 2 g. of vanillin and the urine excreted during the following 5 hr. was collected. The urine (*ca.* 800 ml.) was filtered through cotton wool, made strongly acid with conc. HCl and mixed with 200 ml. alcohol containing 1 g. of β-naphthylhydrazine hydrochloride. The mixture was kept at 0° overnight, during which time *glucurovanillin-β-naphthylhydrazone* crystallized as flat needles set in rosettes (yield, 2 g.). It was purified by dissolving in methyl alcohol, in which it formed an orange-red solution, and then adding a few drops of water whereby the hydrazone crystallized. It formed pinkish flat needles, M.P. 179° and $[\alpha]_D^{20°} -78{\cdot}9°$ ($c=0{\cdot}5$ in methyl alcohol). (Found: C, 59·0; H, 5·6; N, 5·7; OMe, 6·7 %. $C_{24}H_{24}O_8N_2,H_2O$ requires C, 59·3; H, 5·4; N, 5·8; OMe, 6·4 %.) It was very sparingly soluble in water, slightly acid to wet litmus paper and, on heating with 2*N* HCl, a black intractable residue and a reducing solution smelling of vanillin were produced. It gave an intense naphthoresorcinol reaction for glucuronic acid, and on exposure to light, it gradually turned light brown in colour. In this colour change on exposure to light, it resembles vanillin β-naphthylhydrazone which, according to Rothenfusser [1907] turns from a flesh colour to red, an observation which we have confirmed.

Glucurovanillin 2:4-dinitrophenylhydrazone

Six rabbits were each given 2 g. of vanillin. The urine (960 ml.) was collected during the next 5 hr., acidified with HCl and filtered. 2 g. of 2:4-dinitrophenylhydrazine, and sufficient sulphuric acid to convert it into the sulphate, were dissolved in 40 ml. alcohol and the solution was added to the urine. The mixture was kept 20 min. and then the red precipitate which had formed was filtered at the pump, washed with dilute H_2SO_4 and dried for 2 days in a desiccator (yield crude, 3·5 g.). The substance was now dissolved in hot dioxan and the solution was filtered to free it from a brown insoluble residue. Ligroin was added to the filtrate to produce a faint turbidity and the precipitation of the hydrazone was completed on keeping in the refrigerator. The precipitate was collected and recrystallized from dioxan-ligroin, from which it formed very dark red thick plates. At this stage it appeared to melt at 154°, but after drying *in vacuo* at 60°, it decomposed at 200° after shrinking at 150°. It showed $[\alpha]_D^{21°} -68{\cdot}2°$ ($c=1$ in dioxan). The *glucurovanillin-2:4-dinitrophenylhydrazone* was slightly soluble in water, insoluble in ligroin, moderately soluble in alcohol and easily soluble in dioxan. It gave the naphthoresorcinol reaction for glucuronic acid. (Found:[1] OMe, 6·3 %. $C_{20}H_{20}O_{12}N_4$ requires OMe, 6·1 %.)

The above hydrazone (0·2 g.) was boiled with 25 ml. 2*N* HCl for 30 min. The resulting solution smelt strongly of vanillin. The whole was kept overnight at 0° and then the red precipitate present was filtered off. The filtrate, which smelt of vanillin, reduced Fehling's solution and gave an intense naphthoresorcinol reaction for glucuronic acid. The residue (0·1 g.) of vanillin 2:4-dinitrophenylhydrazone was recrystallized from dioxan-ligroin and formed red crystals, M.P. with decomposition, 264°, which was not altered on admixture with an authentic specimen. (Found: OMe, 8·9 %. $C_{14}H_{12}O_6N_4$ requires OMe, 9·3 %.)

[1] The analyst reported that this compound was hygroscopic and, on complete drying, tended to explode on combustion. Analysis for C, H and N was therefore unsatisfactory. One analysis, however, gave C, 47·7; H, 4·6 %; calc. C, 47·3; H, 4·0 %.

Experiments on the demethylation of vanillic acid

2 g. of vanillin or vanillic acid were fed to a rabbit, and a 2-day urine collected. The urine after vanillin gave no colour with $FeCl_3$ and was non-reducing to Fehling's and Benedict's solutions. Urine after vanillic acid, however, gave a green colour with $FeCl_3$, indicating the presence of a catechol derivative, but was non-reducing. In each case the urine after filtration was hydrolysed by boiling for 30 min. with 1/5 vol. of conc. HCl. The urine was then extracted with ether in a large continuous extractor. Two extractions were made in each case, the first for 3 hr. whereby the main bulk of the ether-soluble material was extracted and the second for a further 18 hr. Appropriate controls were carried out on normal rabbit urine. A separate experiment showed that vanillic acid was not demethylated by boiling with HCl as used in the above experiments. The results of some of these experiments are given in Table 5.

Table 5. *Experiments on the demethylation of vanillic acid*

		Ferric chloride reaction in neutral solution			
	Urine vol. in 2 days		Hydro-	Ether extract of hydrolysed urine	
Substance fed	ml.	Urine	lysed urine	3 hr.	18 hr.
4 g. vanillin to 2 rabbits	600	—	—*	?	—
4 g. vanillic acid to 2 rabbits	800	Green	Green*	Strong green	Intense green
Diet alone, 2 rabbits	400	—	—	Faint green	—

Diet: cabbage and bran.

— No definite colour. * Reduced Fehling's solution. ? Indefinite.

It can be seen from Table 5 that normal rabbit urine gives a faint catechol $FeCl_3$ reaction. This was not invariably the case, since we have carried out several control extractions and in some cases the catechol reaction was not given. Preusse [1878–9] states that the catechol-like substances in herbivorous urine are derived from tannic acid and similar substances present in the plant material eaten. Attempts were also made to prove the presence of protocatechuic acid in the urine after vanillic acid, by treating the syrupy ether extract (after removal of the vanillic acid by crystallization) with bromine in acetic acid. A small amount of crystalline material was obtained which was presumably 5-bromo-protocatechuic acid; the amount however was too small for identification so that although protocatechuic acid is probably present it must be considered unproven.

Experiments on the structure of glucurovanillic acid

Preparation of the Ba salt. Rabbits were given 2 g. of vanillin and a 2-day urine was then collected. The normal lead acetate precipitate from the urine was discarded and the basic lead acetate precipitate collected and washed with water. The basic lead precipitate was made into a paste with water and solid barium sulphide was stirred into the mixture until a positive reaction was obtained with sodium nitroprusside. The precipitate of PbS was removed and the solution concentrated *in vacuo* at 50°. The crude Ba salt was precipitated from the concentrated solution with 10 vol. of methylated spirit, centrifuged, washed with alcohol and then dried in a desiccator. The salt was purified by dissolving in water and precipitating with alcohol. An alternative method was

also used, in which the lead from the basic lead precipitate was removed by H_2S and the resulting solution neutralized with $BaCO_3$ for preparation of the Ba salt. The crude Ba salt, after two or three precipitations from water by alcohol, had $[\alpha]_D - 18$ to $20°$ (the pure salt has, according to Kotake [1905], $[\alpha]_D - 38°$ in water) with a Ba content of 28–32 % (theory, 28·6 %). Several samples of the crude salt were prepared, but only the description of the sample which was methylated need be given. 26 g. of this sample were obtained after feeding 20 g. of vanillin. It was slightly reducing and contained about 30 % Ba glucurovanillate, 22 % Ba vanillate, 5 % Ba salt of glucurovanillin, 0·6 % ethereal sulphate (as SO_4) together with a large amount of Ba acetate and some glucuronate. The specific rotation of the crude salt was about $-10°$ in water. Both acetic and vanillic acids could be extracted with ether from an acidified aqueous solution of the salt. Attempts to purify the salt by fractional precipitation from aqueous solution by alcohol were unsuccessful. The purest fraction obtained had $[\alpha]_D - 23°$ in water. Attempts to prepare a pure Ba salt were therefore abandoned and the crude salt was methylated.

Methylation of the Ba salt. The crude salt (20 g.) was dissolved in water (100 ml.) and acetone added to slight turbidity. The solution was then treated with 50 ml. methyl sulphate and 120 ml. 30 % NaOH added dropwise during 1 hr. and the mixture stirred for 4 hr. at 35°, finally at 75° for 1 hr. The resulting solution was then cooled in ice and $5N$ H_2SO_4 was added until the reaction was just alkaline to litmus. The solution was filtered, the filtrate concentrated *in vacuo* to small bulk and remethylated with methyl sulphate and alkali as before. The solution was this time made slightly acid to Congo paper with $5N$ H_2SO_4 and treated with methylated spirit to precipitate Na_2SO_4. The latter was filtered off, the filtrate made faintly alkaline and evaporated *in vacuo* to small bulk. The residue was now acidified and exhaustively extracted with $CHCl_3$. The extract was dried over anhydrous Na_2SO_4 and then concentrated *in vacuo* to a syrup (6 g.). A small amount of acetic acid in the syrup was removed by dissolving in water and concentrating *in vacuo*. The syrupy product was now partially crystalline.

This product was taken up in water and on further dilution with water, a crystalline precipitate was formed and filtered off. This precipitate (2·1 g.) was recrystallized from hot water (needles) and identified as veratric acid, M.P. and mixed M.P. 180°. (Found: OMe, 33·9 %. $C_9H_{10}O_4$ requires OMe, 34·1 %.)

The filtrate from the veratric acid was now concentrated to a syrup (3·5 g.) and given five treatments with methyl iodide and silver oxide. The product was a partially crystalline syrup (3 g.) from which the crystalline material (0·86 g.) was separated by recrystallization from ether-light petroleum. These crystals were the methyl ester of *2:3:4-trimethyl*-o-*methoxy*-p-*carbomethoxyphenyl*-β-d-*glucuronide* (dimethyl ester of trimethylglucurovanillic acid), M.P. 137° and $[\alpha]_D^{22°} - 86·05°$ ($c = 0·5$ in $CHCl_3$). (Found: C, 54·9; H, 6·1 %; OMe, 45·5. $C_{19}H_{26}O_{10}$ requires C, 55·1; H, 6·3; OMe, 44·9 %.)

After removal of the crystalline material, the residual solution was evaporated to a syrup (2 g.). It was distilled in a vacuum and two fractions were obtained. The first fraction (0·4 g.) distilled at bath temperature 140–150°/2 mm. and had $n_D^{21°} = 1·5235$. This fraction crystallized on adding water and after recrystallization from water was identified as methyl veratrate, M.P. 59°: (Found: OMe, 48·5 %. $C_{10}H_{12}O_4$ requires OMe, 47·4 %.) The aqueous filtrate from the methyl veratrate on evaporation gave a syrup (47 mg.) with $n_D^{19°} = 1·4680$ and was probably the methyl ester of 2:3:4-trimethyl-αβ-methylglucuronide. It formed an amide with methyl alcoholic ammonia, M.P. 176° and $[\alpha]_D + 40°$ in water, but the amount

was too small for fuller investigation. The second fraction (1·42 g.; OMe, 46·5 %) which distilled at bath temperature 210–230°/2 mm., crystallized spontaneously on keeping and proved to be the dimethyl ester of trimethylglucurovanillic acid, M.P. 135° and $[\alpha]_D^{21°} -83·1°$ ($c=2$ in $CHCl_3$).

Hydrolysis of the dimethyl ester of trimethylglucurovanillic acid

The ester (0·86 g.) was hydrolysed by heating with 35 ml. of a solution of 2 % hydrogen chloride in dry methyl alcohol, in a sealed tube at 85–90° for 9 hr. The solution was then neutralized with silver carbonate, filtered through a pad of charcoal and evaporated *in vacuo* to a syrup showing $[\alpha]_D^{20°} +48·2°$ ($c=1$ in $CHCl_3$). The syrup was dissolved in hot acetone and, on dilution with water, 113 mg. of the unhydrolysed ester, M.P. 136° were recovered. The filtrate from this was evaporated to a syrup (637 mg.) which was distilled in a high vacuum. Two fractions were collected, (*a*) 432 mg. at bath temperature 120°/2 mm.; $n_D^{20°}=1·4880$; OMe, 52·8 %; $[\alpha]_D^{20°} +68·7°$ ($c=1$ in $CHCl_3$) and (*b*) 97 mg. at bath temperature 140–150°/3 mm.; $n_D^{20°}=1·4949$; OMe, 53 %. The fractions were practically identical in composition, and were mixtures of methyl vanillate (25 %) and the methyl ester of 2:3:4-trimethyl methylglucuronide (75 %), the percentages being calculated from the methoxyl value.

The identification of these esters was carried out as follows: 221 mg. of fraction (*a*) were dissolved in 3 ml. of dry methyl alcohol and the solution was saturated with dry ammonia at 0°. After 24 hr. at 0° the solution was evaporated in a desiccator, leaving a partially crystalline residue. The latter was dissolved in acetone and treated with ether until crystals (needles) appeared. The crystals (121 mg.) proved to be the amide of 2:3:4-trimethyl-α-methylglucuronide. (Found: OMe, 49·8 %. $C_{16}H_{19}O_6N$ requires OMe, 49·8 %.) M.P. and mixed M.P. 182° (an authentic sample, M.P. 183° was prepared from glucurone) and showing $[\alpha]_D^{20°} +135°$ ($c=1·1$ in water) (Smith [1939] gives M.P. 183° and $[\alpha]_D +137·5°$ in water).

The filtrate after separation of the above amide was now evaporated to a syrup (100 mg.). It was distilled in a vacuum and the distillate ($n_D^{18°}=1·5229$) crystallized on keeping. The crystals were identified as methyl vanillate and after recrystallization from water (needles) had M.P. and mixed M.P. 63°. (Found: OMe, 32·8 %. $C_9H_{10}O_4$ requires OMe, 34·1 %.) It gave, on heating in water with a drop of Millon's reagent, an intense purple colour. It has been shown by Thorpe & Williams [1937] that, of a large number of phenols tested, only those containing the vanillin grouping, i.e. vanillin and vanillic acid, give a purple colour with Millon's reagent. A control experiment showed that methyl vanillate did not form an amide in methyl alcoholic ammonia.

Discussion

Since the present experiments suggest that with some compounds conjugation may precede an oxidation, they have an important bearing on what is generally accepted as the theory of detoxication. This theory was developed mainly by Sherwin [cf. for example, Sherwin, 1922] and is the accepted version of detoxication as given in text-books of biochemistry. According to this view, when a foreign organic compound finds its way into the body, the organism attempts to deal with it by direct oxidation. If such an attempt fails or only partially succeeds, then the body has recourse to certain special synthetic mechanisms in

order to produce easily soluble and relatively non-toxic substances which are then easily excreted. Taken in its narrowest sense, this theory suggests that the primary process is oxidation, conjugation (i.e. synthetic mechanisms) being a special secondary process and only functioning when oxidation fails.

As a result of our present experiments and suggestions from previous and other work in this Laboratory, we have come to consider the synthetic mechanisms not as complete special processes in themselves functioning because of failure of direct oxidation, but as part of a process leading to 'complete' oxidation. In the so-called 'detoxication' processes, three general mechanisms may be distinguished, namely, partial oxidation, reduction and synthesis, all of which are complementary and interdependent and may represent different stages in the process of 'complete' oxidation. They need not be special mechanisms at all, but mechanisms which are used normally by the body,[1] and which are revealed in exaggerated or possibly modified form when foreign organic compounds have to be dealt with, thus constituting the so-called 'detoxication mechanisms'. According to our view then, when a foreign organic compound enters the body, three general mechanisms, oxidation, reduction and synthesis come into play either simultaneously or consecutively and these are parts of the process of complete oxidation. The chemical nature of the compound, however, appears to be the deciding factor in the speed of the 'complete' oxidation or of any stage of it, and some of the compound or intermediate compounds in the oxidation (i.e. reduction or partial oxidation products or conjugates) may escape via the kidney if the speed is low. With some compounds the speed of the 'complete' oxidation would be so great that very little of them or their intermediate metabolites would have time to escape *via* the kidney. The order in which the three mechanisms mentioned above would occur (if applied in stages) will depend on the chemical structure of the compound being oxidized. It may be that the compound would first be partially oxidized, then conjugated and the conjugate may then be amenable to further oxidation, or perhaps the compound is first reduced, then conjugated and then oxidized and so on.

If, for example, vanillin is considered in relation to the metabolisms of vanillic and protocatechuic acids, it is probable that if it remained long enough in the body it would be converted into catechol thus: vanillin $\xrightarrow{\text{conjugation}}$ conjugated vanillin $\xrightarrow{\text{oxidation}}$ conjugated vanillic acid $\xrightarrow{\text{oxidation, i.e. demethylation}}$ conjugated protocatechuic acid $\xrightarrow{\text{oxidation, i.e. decarboxylation}}$ conjugated catechol. The benzene ring in catechol would then be opened by an oxidation at a point between the two *ortho*-hydroxyl groups and then finally be converted into CO_2 and H_2O.

Summary

A quantitative study has been made of the fate of vanillin and vanillic acid in the rabbit. In each case, about 83 % of the dose fed was accounted for by the material excreted in the urine.

In the case of vanillin, 69 % was oxidized to vanillic acid, 44 % being free vanillic acid and 25 % conjugated; 14 % of the vanillin was excreted unoxidized but conjugated mainly as glucurovanillin. Of the vanillin accounted for 8 % was excreted as ethereal sulphate and 31 % as glucuronide. Two conjugated glucuronides were excreted, namely glucurovanillin and glucurovanillic acid.

[1] Quick [1927] also expresses the opinion that detoxication mechanisms may be normally occurring processes as opposed to 'special' mechanisms.

The former was isolated as the β-naphthylhydrazone and 2:4-dinitrophenylhydrazone. Methylation of crude barium glucurovanillate gave the crystalline dimethyl ester of trimethylglucurovanillic acid. From a study of the hydrolysis products of this ester, glucurovanillic acid was shown to be *o*-methoxy-*p*-carboxyphenyl-β-*d*-glucopyranuronoside.

In the case of vanillic acid, 56% was excreted as free vanillic acid and 27% conjugated (11% ethereal sulphate and 16% conjugated glucuronide). A small amount, probably not more than 5% was demethylated, producing probably protocatechuic acid.

The mechanism of the oxidation of vanillin and vanillic acid has been discussed and it is suggested that conjugation of these substances may be a necessary stage in their oxidation.

The bearing of these results on the theory of detoxication has also been discussed.

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Appendix

A colour reaction for p*-hydroxy- and* p*-methoxy-benzaldehydes*

Since aromatic aldehydes are usually oxidized in the animal body to the corresponding acids, it was important to find out easily whether this oxidation was complete as judged by the presence or absence of the aldehyde in the urine after its administration. The following colour reaction was found which distinguished between the aldehyde and the corresponding acid.

The test is carried out as follows. To a little of the solution or urine containing the aldehyde, a small amount of solid naphthoresorcinol is added followed by a few drops of alcohol, then an equal volume of conc. HCl and the whole mixed. If an aromatic aldehyde containing a *p*-OH or *p*-OMe is present, a strong red colour develops immediately, followed by a dark red precipitate if much of the aldehyde is present. The following compounds gave the test *p*-hydroxy-, 2:4-dihydroxy-, anisic, protocatechuic, and veratric aldehydes, vanillin, piperonal (methylene-3:4-dioxybenzaldehyde), and anisaldehyde sodium bisulphite. Vanillin β-glucoside also gave the test, but here the colour came up more slowly. The corresponding acids did not give a colour. Cinnamic aldehyde, but not cinnamic acid, also gave the reaction. Salicylaldehyde and *o*-methoxybenzaldehyde gave orange-red and scarlet colours respectively, followed by precipitates. Benzaldehyde, *p*- and *m*-nitrobenzaldehydes and *m*-hydroxybenzaldehyde did not give a red colour, nor did ketones and aliphatic aldehydes. With acetylvanillin the reaction was negative, but became positive after alkaline hydrolysis. *p*-Dimethylaminobenzaldehyde gave a blue-green colour followed by a blue-green precipitate. If α- or β-naphthol were substituted for naphthoresorcinol no colour was obtained and with resorcinol or orcinol only a faint red colour was produced. It will be remembered that naphthoresorcinol and HCl are reagents in a well-known test for certain sugars particularly the uronic acids [cf. Tollens, 1908; Rorive & Tollens, 1908; Neuberg & Kobel, 1931], but in the sugar test the solution has to be boiled, whilst the present one is carried out without heating.

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132. STUDIES ON IRON IN PLANTS WITH SPECIAL OBSERVATIONS ON THE CHLOROPHYLL : IRON RATIO

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The starting point for the investigations about to be recorded was that one of us [Hill, 1937; 1938] found that chloroplasts, which he was able to separate from the cells of green leaves, produced oxygen if iron salts were added to the medium in which they were suspended.

The connexion between chlorosis and iron deficiency in green plants is well known [cf. Czapek, 1920]. Investigations by Oserkowsky [1933] show that not all of the iron is available during the formation of chlorophyll. He found that one of the factors deciding the development of chlorosis in leaves is lack of iron in the earlier part of the season. Oserkowsky differentiates between 'active' and total iron; active—we would call it available—iron is described by him as the part of the iron in dried leaves obtainable by extraction with *N* HCl. The chlorophyll content of chlorotic leaves is proportional to the active iron and not to the total amount of that mineral present.

In the present work measurements of iron and chlorophyll simultaneously in normal green leaves of different plants have been made. In several cases the chlorophyll : iron ratios throughout a season were followed. We have been concerned with measurements of the total iron in the plant tissue, as the nature of the iron compounds present is still obscure. It will be seen that the iron compounds fall into three main classes based on solubility.

Methods

The chlorophyll was estimated by measuring the degree of extinction of a band in the red part of the spectrum of acetone extracts of fresh leaves. We measured the extinction at a wave-length of 6600 Å. using the spectrophotometer. The actual point where the greatest absorption could be found was always chosen for measurement. This was between 6550 and 6650 Å.

Control experiments showed that it made no difference if the acetone extracts were prepared in diffuse daylight or in the dark room illuminated with a green-coloured lamp.

The molecular absorption coefficient $\log_{10}(I_0 \times I)$ for chlorophyll in acetone was taken as $0{\cdot}46 \times 10^{-3}$ [see also Hill & Scarisbrick, 1940].

The measurement of the iron followed the principles laid down by Hill [1930]. After ashing of dried leaves at low temperature the inorganic residue was boiled in *N* HCl following McCance & Shipp [1933]. The additional manipulations for complete extraction suggested by McCance *et al.* [1936] could be omitted provided that the temperature of ashing was kept sufficiently low. $\alpha'\alpha'$-Dipyridyl in excess was added followed by 4 mol. of sodium acetate for each mol. HCl, and a

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reducing agent added in solid grains, usually sodium hydrosulphite. The developing colour was compared with that of standard solutions of different amounts of reduced iron and $\alpha'\alpha'$-dipyridyl. These solutions were standardized by titanium titration.

The reagents used were always tested for the presence of iron and were found to be practically free. The sodium acetate solutions were boiled and filtered, the HCl stock was specially chosen; the sodium hydrosulphite contained on the average 0·02 μg. iron/mg.

Both wet and dry methods of ashing were used. The dry ashing method was found reliable for dried leaves but not for leaf extracts unless a platinum crucible was used. In the case of fluids containing biological material a large surface of dried residue was in contact with the porcelain crucible, and, as it was invariably alkaline, it attacked the crucible and set free iron which was soluble in acid afterwards. When phosphorus was measured, the method of Fiske & Subbarow [1925] was used.

Several estimations of chlorophyll and iron respectively on different samples of the same material were made. Values were only taken into account when the different estimations were in agreement within the limits of experimental errors.

If the midrib and veins of the leaves or leaflets were big enough to have appreciable influence on the results—as in the case of horseradish or chestnut—these parts of the plant tissue were removed before the chlorophyll and iron were examined.

The dry weight of the leaves was determined and usually the ash. Both will be expressed as percentage of the fresh weight. Chlorophyll and iron will be given in terms of molarity $\times 10^{-3}$, for this makes easier a comparison between the concentrations of the two. The molarity is expressed per g. fresh leaf as we do not know yet how much of the iron and the chlorophyll are actually in solution or are incorporated with the solid parts of the plant tissues. Since the dry weight and ash are measured at the same time and recorded in the text, the results can be calculated on any other basis.

The time of the year and the number of leaves per g. will give an idea of the seasonal stage of the plants in which the different measurements were taken.

I. Seasonal observations

The iron and chlorophyll contents of green leaves and leaflets throughout a season have been determined in:

(*a*) Elder (*Sambucus nigra*).
(*b*) Chestnut (*Hippocastanum*).
(*c*) Dead nettle (*Lamium album*).
(*d*) *Claytonia perfoliata*.

(*a*) *Elder*

It will be observed in Table 1 that in May, during the time of the most active growth, the chlorophyll : iron ratio rises, but it returns in June to the values measured in April, and even the appearance of the iron-containing fruits does not influence the ratio to any great extent.

Some special observations on flowers and fruits were made. Though both these organs of the elder contained iron, their provision did not affect the iron content of the leaflets (Tables 1*a* and 1*b*).

Table 1. *Chlorophyll and iron in elder throughout a season*

Date	No. of leaflets per g.	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron	Special remarks
4. iii. 37	70	22	—	2·1	1·5	1·4	—
16. iii. 37	45	22	—	2·0	1·1	1·8	—
2. iv. 37	28	23	—	1·6	1·3	1·2	—
16. iv. 37	12	21	—	3·2	0·8	4·0	—
7. v. 37	3–20	21	1·7	3·0	0·4	7·5	—
12. v. 37	2½	19	1·6	3·0	0·4	7·5	—
19. vi. 37	2	22	2·5	3·6	0·7	5·1	In flower
7. viii. 37	1	24	3·7	4·0	1·0	4·0	Fruits appear
26. viii. 37	1–1½	21·5	3·2	3·4	0·5	6·8	—
8. ix. 37	{Green 1–1½	23	—	4·0	0·2	20·0	—
	{Yellow 1–2	14	2·5	0·2	0·2	1·0	—

Table 1*a*. *Iron in leaflets and corollae of elder*

19. vi. 37	Green leaflets	Corollae
Dry weight %	22·4	21·0
Ash %	2·5	1·9
Iron, $M \times 10^{-3}$	0·67	0·42
Chlorophyll, $M \times 10^{-3}$	3·64	—

Table 1*b*. *Iron in leaflets and fruits of elder*

	7. viii. 37		8. ix. 37	
	Green leaflets	Fruits	Green leaflets	Fruits
Dry weight %	24	17·5	23	8·4
Ash %	3·6	1·0	—	4·3
Iron, $M \times 10^{-3}$	1·0	0·3	0·2	0·1
Chlorophyll, $M \times 10^{-3}$	4·0	0·4	4·0	—

The absolute iron content per individual fruit remained constant (Table 1*c*).

Table 1*c*. *Iron in individual berries of elder*

	7. viii. 37	8. x. 37
Average weight of berry, mg.	100	350
Dry weight %	17·5	8·4
Average weight of iron per berry, μg.	11·9	11·9

(*b*) *Chestnut*

In chestnut (Table 2) as in elder, it will be seen that the iron precedes the chlorophyll both in appearance and in disappearance.

Table 2. *Chlorophyll and iron in chestnut throughout a season*

Date	No. of leaflets per g.	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron	Special remarks
A. 24. iv. 37	4	19	1·0	2·5	0·25	10·0	—
14. v. 37	¾	19	0·9	2·8	0·25	11·2	—
B. 17. v. 37	1	—	—	—	0·5	—	In flower
9. viii. 37	½	30	2·5	6·8	1·2	5·7	Developing fruits
22. ix. 37	¾	35	3·4	6·8	0·4	17·0	Fruits fully developed and shedding
27. x. 37	1	32	3·2	1·0	0·5	2·0	Yellow leaflets

Two different trees were investigated: A and B. A was much later in seasonal development.

The blossoms again contain quite an appreciable amount of iron, and this has no effect on the iron content of the leaflets (Tables 2*a* and 2*b*).

Table 2*a*. *Iron in leaflets and corollae of chestnut* (*tree B*)

B. 14. v. 37	Green leaflets	Corollae	Stamens, pollen removed
Dry weight %	—	10·4	12·7
Ash %	—	1·0	0·7
Iron, $M \times 10^{-3}$	0·45	0·2	0·1
Iron, % of ash	—	4·7	1·0

Table 2*b*. *Iron in leaflets and young fruits of chestnut* (*tree B*)

B. 9. viii. 37	Green leaflets	Young fruits
Dry weight %	30	40
Ash %	2·5	1·5
Iron, $M \times 10^{-3}$	1·2	0·4
Iron, % of ash	2·7	1·4

In contrast to what was found in the elder, the appearance of the fruit is accompanied by a considerable fall in the iron (Tables 2*a* and 2*b*).

The distribution throughout the fruit was investigated (Table 2*c*).

The brown 'shells' are comparatively rich in iron; next to the plumules contain more iron than the rest of the seed per g.

Table 2*c*. *Iron in fruit of chestnut*

22. ix. 37	Whole fruit	Husk one pericarp	Two chestnuts	Shells	Two plumules	Rest of embryo
Average weight of fruit, g.	67	25	42	4·8	0·3	37
Dry weight %	57	45–63	59	73	36	57
Ash %	2·9	2·9–4·1	2·5	4·4	2·2	2·6
Iron, μg./g. wet weight	8·4	6·25	9·5	44	15	5
Iron, μg./fruit	560	160	400	210	5	190

(*c*) *Dead nettle*

In the case of dead nettle, plants growing in different localities had the same iron content and chlorophyll : iron ratio.

Again the iron precedes the chlorophyll in both accumulation and removal (Table 3). The provision of the flowers with iron (Table 3*a*) does not affect the iron content of the leaflets any more than it did in elder and chestnut.

Table 3. *Chlorophyll and iron in dead nettle throughout a season*

Date	No. of leaves per g.	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron	Special remarks
8. iv. 37	6	18	—	3·4	0·8	4·25	—
19. iv. 37	5	17	—	3·8	0·5	7·6	—
19. v. 37	4	17	2·8	3·6	1·4	2·6	In flower
10. viii. 37	{Green 7	22	3·5	3·8	1·3	2·9	—
	{Yellow 7	22	4·3	1·4	0·7	2·0	—

Table 3*a*. *Iron in leaflets and corollae of dead nettle*

19. v. 37	Green leaflets	Corollae flower	Stamens
Dry weight %	17	10	9
Ash %	2·8	1·1	1·7
Iron, mol. $\times 10^{-3}$	1·4	0·4	0·2

(*d*) Claytonia perfoliata

The inflorescent leaves, which differ anatomically from the radical leaves and are only half the size, have the same iron content and chlorophyll : iron ratio. The iron precedes the chlorophyll in the leaves in both accumulation and removal (Table 4).

Much of the iron which the inflorescent leaves lose in the later part of the season can be found again in the seed.

The seed of *Claytonia* was collected in June. Each inflorescence produced about 45 seeds. If the iron found in 45 seeds is added to the amount found in the leaf, the iron content rises from $0{\cdot}2 \times 10^{-3}$ to $0{\cdot}4 \times 10^{-3}$ *M*, nearly as high as in April, when the iron was $0{\cdot}5 \times 10^{-3}$ *M*.

Table 4. *Chlorophyll and iron in* Claytonia perfoliata *throughout a season*

Date	No. of leaves per g.	Dry weight %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	$\frac{\text{Chlorophyll}}{\text{Iron}}$	Special remarks
27. ii. 37	70	7	1·2	1·0	1·2	—
14. iv. 37	15	7	2·4	0·5	4·8	In flower
1. vi. 37	2–3	6	1·8	0·2	9·0	Seed

The seed had a dry weight of 76·6 and 1·9 % ash. In 230 mg. seed (66 seed $\approx$ 100 mg.) were found 27 μg. iron.

Four inflorescent leaves containing 20 μg. iron and weighing 1·513 g. (iron $0{\cdot}2 \times 10^{-3}$ *M*) have 180 seeds $\approx$ 119 mg. 119 mg. seed contain 14 μg. iron. Adding the iron content of the seed to that of the leaves four inflorescent leaves contain 34 μg. iron or 22·5 μg. iron/g. fresh weight. This would correspond to a molarity of $0{\cdot}4 \times 10^{-3}$ iron.

Iron content of chloroplasts

A comparison has been made in the case of *Claytonia* between the iron content of the whole leaf and that of the isolated chloroplasts. Suppose the quantity of iron in the whole leaf is 300; if this iron were evenly distributed through the leaf, the amount present in the chloroplasts would be represented by 8; the amount actually found was 31. This is the first time that the high iron content of chloroplasts as compared with other parts of the cell has been demonstrated by chemical measurement. It was hitherto only assumed on the basis of histological staining methods.

II. Leaves of evergreens

Measurements were made of the chlorophyll : iron ratio in evergreen plants, younger leaves being compared with older ones of the same individual plant. It

was generally found that the chlorophyll : iron ratio was higher in older than in younger leaves. The plants investigated were:

(*a*) *Euonymus japonicus.*
(*b*) Japanese laurel (*Aucuba japonica*).
(*c*) Box (*Buxus sempervirens*).

(*a*) Euonymus

Leaves of *Euonymus* of different kinds were investigated in May 1937. Dark green leaves surviving from 1936 and dark green ones from 1937 were chosen, and also the very yellow leaves on the end of the twigs and the light green ones below them which were just changing from the yellow state into the normal dark green appearance.

The difference in dry weight between green and yellow leaves may be due in part to differences in starch content.

After a sunny day (31. v. 37) four yellow and four green leaves of equal length (4–5 cm.) were picked, weighed and extracted with alcohol. The yellow leaves weighed 0·647 g., the green ones 0·813 g. Whereas the residue contained plenty of starch in the case of the latter, no trace of starch could be found in the residue of the yellow samples.

Whereas the chlorophyll : iron ratio in the 1936 leaves was about ten times as great as in the youngest of those of 1937, it will be noted that the phosphorus : iron ratio is the same in all leaves, i.e. about 70 (Table 5). It might be suggested that phosphorus was connected with the carbohydrate metabolism in the absence of chlorophyll. The state of the yellow leaves may recall the findings in haemosiderosis in man, in which an enormous accumulation of iron in the liver goes hand in hand with a lack of respiratory pigment.

Table 5. *Chlorophyll, iron and phosphorus in* Euonymus

Date	Kind of leaves	No. of leaves per g.	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron	Phosphorus, $M \times 10^{-3}$	Phosphorus / Iron
24. v. 37	Dark green of 1936	2	29	1·9	3·0	0·2	15·0	13·9	69
24. v. 37	Dark green of 1937	3	25	2·5	2·2	0·4	5·5	29	72
21. v. 37	Light green of 1937	7	17	2·2	0·4	0·5	0·8	—	—
21. v. 37	Yellow of 1937	7	19	1·5	0·2	0·7	0·3	47·5	68

Keilin [1925] has shown *Euonymus* to be rich in haematin. We measured the actual amount in our leaves but found only a concentration of $0{\cdot}6 \times 10^{-4} M$ of haematin iron. Though the enormous accumulation of iron cannot be accounted for as haematin, it is significant to find so much just before chlorophyll is formed.

(*b*) *Japanese laurel*

In Japanese laurel the distinction between the light green leaves of the current year and the dark green ones of the previous season was obvious in the month of April and similar, though less marked, differences in the chlorophyll and iron distribution were found as in *Euonymus* (Table 6).

Table 6. *Chlorophyll and iron in Japanese laurel, 26. iv. 37*

Kind of leaves	No. of leaves per g.	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron
Young	15	29	1	0·4	0·2	2·0
Old	$\frac{1}{4}$	31	2	2·6	0·3	8·7

(*c*) *Box*

In this plant (Table 7) it will be noted again that iron accumulates before the chlorophyll. Whereas in May the old leaves contained more iron than the new ones, in August the relation is reversed; most of the iron is now to be found in the leaves of 1937 and least in those of 1933.

Table 7. *Chlorophyll and iron in box*

Date	Kind of leaves	No. of leaves per g.	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron
20. v. 37	Leaves of 1933–36	25	44	0·7	3·4	0·5	6·8
	Leaves of 1937	25	27	1·2	1·4	0·3	4·7
11–14. viii. 37	Leaves of 1933	22	43	2·8	1·5	0·25	6·0
	Leaves of 1934–36	25	44	2·8	4·0	0·3	13·3
	Leaves of 1937	31	44	2·3	3·0	0·5	6·0

III. Single measurements of chlorophyll : iron ratio in several plants

(*a*) Tulip (*Tulipa*).
(*b*) Horseradish (*Cochlearia armoracia*).
(*c*) Spinach (*Spinacia oleracea*).
(*d*) *Tropaeolum*.
(*e*) Garlic mustard (*Alliaria officinalis*).
(*f*) Golden elder (*Sambucus nigra*, golden variety).

(*a*), (*b*) Very little iron was to be found in tulips, though it must be kept in mind that the high water content of this plant may be responsible (Table 8). The blossoms and pistils were also investigated. It can be seen that the iron is fairly equally distributed throughout the different organs, if the ash as a whole is taken as a comparative basis.

Table 8. *Chlorophyll and iron in tulip, 16. v. 37*

Organ	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron	Iron, % of ash
Leaf (1/20 per g.)	11·7	0·89	1·0	0·29	3·4	1·8
Perianth leaves	8·1	0·34	—	0·11	—	1·7
Pistil, pollen removed	9·5	0·58	—	0·15	—	1·4

Table 9. *Chlorophyll and iron in horseradish, 12. vi. 37*

Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	Chlorophyll / Iron
19	2·1	3·2	0·45	7·1

(*c*) In spinach, as in tulip, a low iron content goes hand in hand with a low dry weight (Table 10).

Table 10. *Chlorophyll and iron in spinach, 21. x. 37*

Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	$\frac{\text{Chlorophyll}}{\text{Iron}}$
10–15	2	1·9	0·2	9·5

The chlorophyll : iron ratio is particularly high. It is difficult to understand why it has been recommended as an iron-containing food with an iron content of only $0{\cdot}2 \times 10^{-3}\,M$. But it may be noted that large amounts of spinach leaves have to be used in preparing a dish of the cooked vegetable.

Table 11. *Chlorophyll and iron in* Tropaeolum, *30. x. 37*

Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	$\frac{\text{Chlorophyll}}{\text{Iron}}$
14·5	1·4	2·8	0·3	9·3

(*d*) As we had always observed that the iron content of the leaves falls before the chlorophyll disappears, we used the leaves of *Tropaeolum* to find out if the removal of iron is a necessary condition for the yellowing of leaves. We left the leaves of *Tropaeolum* in water and observed their yellowing. We then measured the iron content of the leaf as a whole and of the green centre of the leaf which we separated from the yellow border. We also determined the concentration of iron in the stalks. Whereas the chlorophyll content decreased from $2{\cdot}8 \times 10^{-3}\,M$ in 9 days to $0{\cdot}26 \times 10^{-3}\,M$, the iron content remained constant at $0{\cdot}3 \times 10^{-3}\,M$. The iron in the stalks remained throughout at a molarity of $0{\cdot}1 \times 10^{-3}$. The green centres of the yellowing leaves contained rather less iron than the whole organ, namely $0{\cdot}25 \times 10^{-3}\,M$.

(e, f) Garlic mustard and golden elder

Differences in chlorophyll content were found in leaves of the same plant. This may be due to differences in illumination, leaves growing in the shade being richer in chlorophyll, as if they needed more pigment to make the greatest possible use of what light they can obtain. The difference may be due, on the other hand, to an inherited factor (variety). As an example of the influence of the former factor, garlic mustard was chosen; as an example of the latter, common and golden elder were used.

The specimens of garlic mustard were of two kinds, though they were grown at the same place and picked at the same time. We could differentiate between dark leaves which had grown in the shade and light sun leaves, yet the chloro-

Table 12. *Chlorophyll and iron in garlic mustard (shade and sun leaves) and in golden and common elder*

		No. of leaves per g.	Dry weight %	Ash %	Chlorophyll, $M \times 10^{-3}$	Iron, $M \times 10^{-3}$	$\frac{\text{Chlorophyll}}{\text{Iron}}$
Garlic mustard 4. v. 37	Shade leaves ...	1	11	—	3·6	0·45	8·0
	Sun leaves ...	3	15	—	2·6	0·3	8·7
Elder 7. v. 37		3–20*	21	1·7	3·0	0·4	7·5
Golden elder 5. v. 37		8–16*	18·5	—	1·4	0·45	3·1

* The number of leaves per g. had no great influence on the iron and chlorophyll content.

phyll : iron ratio was the same in all leaves. At the same time an example is given in which leaves of two different varieties of the same species with differing chlorophyll content were compared. Golden elder and common elder differ in pigment but not in iron content (Table 12).

IV. Nature of the plant iron

The observations of Keilin [1925] and Mann [1938] have established the fact that part of the iron is present as haematin, though this proportion is only small. Some of the iron found in plants is soluble in acetone, some in water, some is insoluble in both (Table 13).

Table 13. *Different solubilities of iron found in elder leaflets*

13. v. 37. 10 g. fresh leaflets contain 240 μg. Fe
10 g. fresh leaflets (dry weight 18%) extracted with acetone.
1·2 g. powder are obtained

	Amount of Fe per 10 g. leaflets, μg.
Acetone extract concentrated and ashed	19
42% of powder extracted with water; extract ashed	50
42% of powder ashed after water extraction	168
	237

Table 14. *Iron in water extract of acetone powder of dead nettle leaves*

	μg. Fe
Total iron/g. fresh leaf (17% dry weight)	24
Total iron/g. acetone powder	150
Available iron in 10 : 1 water extract of 1 g. powder	5
Available after 7 min. boiling of extract in HCl	6
Available after 180 min. boiling of extract in HCl	6

We observed several kinds of water-soluble iron in plant extracts and extracts of acetone powders of leaves.

(*a*) A proportion reacts at once with the dipyridyl reagent.

(*b*) A certain proportion reacts after boiling with acid. This can be either an iron protein complex or iron phosphate (Table 14).

(*c*) A proportion varying in amount reacts with dipyridyl only after ashing.

This last fraction seemed to correspond with that which Chibnall & Channon [1929] found in the ether-soluble part of cabbage leaves. We repeated Chibnall & Channon's experiments and confirmed their findings. This fraction may of course result from mechanical adsorption, but even so the adsorption of iron by lecithin should be of biological importance for the transport of this element in the plant.

Summary

The iron and chlorophyll contents of green leaves and leaflets throughout a season have been determined in elder (*Sambucus nigra*), chestnut (*Hippocastanum*), dead nettle (*Lamium album*), and *Claytonia perfoliata*. Iron was always found to precede the chlorophyll both in appearance and disappearance. Blossoms and fruits contain an appreciable amount of iron, yet this fact is without any influence on the iron content of elder and chestnut leaves. 2/5 of the iron of the whole chestnut fruit were found in the woody shells of the embryo. In

Claytonia much of the iron which the green leaves lose in the later part of the season can be found again in the seed. The chloroplasts in *Claytonia* leaves contain four times as much iron as they would be expected to have were the iron equally distributed throughout the leaves. This is the first time that the iron content of the chloroplasts has been chemically measured.

During the development of the leaves of evergreen plants iron storage precedes that of chlorophyll. Green leaves of *Euonymus japonicus* left over from the previous year have a chlorophyll : iron ratio which is ten times as great as that of leaves of the current season. The phosphorus : iron ratio is the same in all leaves. It is suggested that the high phosphorus content in yellow leaves may be connected with carbohydrate metabolism in absence of chlorophyll. Japanese laurel (*Aucuba japonica*) and box (*Buxus sempervirens*) behave in a similar way to *Euonymus*. The chlorophyll : iron ratio was also determined in tulip (*Tulipa*), horseradish (*Cochlearia armoracia*), spinach (*Spinacia oleracea*), *Tropaeolum*, garlic mustard (*Alliaria officinalis*) and golden elder (*Sambucus nigra*, golden variety). The chlorophyll : iron ratio is the same in the dark (shade) leaves and the light (sun) leaves of garlic mustard. Golden elder and common elder differ in pigment but not in iron content.

The iron content in most plants investigated was about a fourth to a tenth of the chlorophyll compared per mol. Some iron in plants is soluble in acetone, some in water, some insoluble in both. Three kinds of water-soluble iron could be observed:

(*a*) A large proportion reacts at once with $\alpha'\alpha'$-dipyridyl.

(*b*) A certain proportion reacts after boiling in acid; this can be either an iron protein complex or iron phosphate.

(*c*) A proportion varying in amounts reacts with $\alpha'\alpha'$-dipyridyl only after ashing.

One of us (R. Hill) was a Beit Memorial Fellow during part of this research. Some of the expenses of the work were defrayed from a research grant from the Ella Sachs Plotz Foundation to H. Lehmann.

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133. THE RELATIONSHIP BETWEEN NITROGEN METABOLISM AND THE DURATION OF THE LARVAL STAGE OF THE DEATH-WATCH BEETLE (*XESTOBIUM RUFOVILLOSUM* DE G.) REARED IN WOOD DECAYED BY FUNGI

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Fisher [1941] has reviewed existing knowledge on food relations of the death-watch beetle. The conclusion of Campbell & Bryant [1940], that fungus benefits the insect largely because of the depreciation which it induces in the mechanical strength of the substrate, is accepted, but the suggestion is made that the insect may nevertheless benefit in some more direct manner from the presence of fungus or fungal remains in its substrate. For example, fungal mycelium may be the means of concentrating certain ferments in the larval gut or, alternatively, it may provide a rich source of nitrogen. It is known from the work of Findlay [1934] that fungi growing in wood are capable of accumulating N to the extent that their content of that element may be as much as forty times as great as that of their woody substrate. Fisher [1941] therefore draws attention to the work of Becker [1938], in which conclusive evidence is given that the addition of an organic source of N (peptone) to wood results in a marked increase in the growth rate of the larvae of *Hylotrupes bajulus* boring in it. Becker [1938] made the further observation that fungal attack in wood can also favour the development of *Hylotrupes* under certain conditions, and suggested the possibility that the controlling factor here is the enrichment of the N compounds in the wood. The mechanism of the enrichment process is not discussed, but the biological observations of Becker [1938] form an important contribution to the N metabolism of wood-boring insects, and they have prompted the present re-examination of the relationship between the N metabolism of *Xestobium* and the duration of its larval stage in wood which has been decayed in known amounts by fungi.

In contrast to carbohydrates, N compounds usually form a very small proportion of wood. The total N content of the oak wood used in this and previous work was of the order of 0·2%, and since the N content of the *Xestobium* larva has been found to be 3·91% [Campbell & Bryant, 1940], it is obvious that a considerable amount of the substrate must be comminuted by each individual before its N requirements for body-building purposes can be satisfied. Pupation presumably cannot normally take place until these requirements are met, and there would thus appear to be good grounds for the belief that any agency which is effective in increasing the rate of supply of N for conservation purposes

to the growing larva will tend to shorten the larval stage. It is important to note that when Becker [1938] added peptone to wood infested by *Hylotrupes* he found that the more rapid larval growth which ensued was caused by a greater intake of protein and not by a greater intake of wood. Treated wood was not disintegrated to a greater extent than wood to which no peptone was added. On the other hand, in wood previously decayed by fungi the larvae grew at a greater rate, but the extent of damage caused in the substrate by boring on the part of the larvae was greater than in sound, control samples of the same wood. The full significance of these findings will be discussed later.

From an investigation of the N contents of sound and decayed woods, Huntgate [1940] has concluded that there is little change in the total N content during fungal decay, and it is obvious that if this conclusion holds for the decayed oak wood used in the previous work on *Xestobium* carried out in this laboratory [Fisher, 1935; 1937; 1940; 1941; Campbell & Bryant, 1940] the progressive shortening of the life cycle which has been found to accompany increases in the extent of fungal decay in the substrate cannot be based on any simple enrichment process of the nitrogenous components of the wood, although the direct bearing of N metabolism on the duration of the larval stage cannot be denied.

Experimental

The present study has fallen naturally into two parts, (1) the effect of fungal decay on the N content of oak sapwood, and (2) the N economy of *Xestobium* larvae reared in decayed oak sapwood under controlled conditions. All N determinations were carried out according to the modified Kjeldahl procedure of Stubblefield & De Turk [1940]. 2 g. (air-dry) samples of wood were digested with 10 g. K_2HPO_4, 6 g. $Fe_2(SO_4)_3$, 0·6 g. Hg and 25 ml. H_2SO_4: digestion was usually complete within 45 min. The method was preferred to that used previously [Campbell & Bryant, 1940].

The effect of decay by Phellinus cryptarum *Karst. on the N content of oak sapwood*

The material consisted of remnants of samples of sound wood, and of wood decayed to known losses in weight by the fungus, used in previous studies [Fisher, 1941; Campbell & Bryant, 1940]. The greater part of five of the samples of decayed wood had been used by Fisher [1941] for beetle infestation. The samples which had lost 22·65, 30·8 and 51·20 % of their respective dry weights owing to decay were not so used, but N values have been found for them for present purposes (Table 1).

With one exception, the results in Table 1 show that each sample of decayed wood has a higher N content than sound wood of the same origin. At the same time, in terms of original sound wood, N is progressively removed by the fungus as the extent of decay increases. This latter observation is explained by the fact that a considerable weight of fungal mycelium containing a relatively high proportion (0·68 %) of N derived from the wood [Campbell & Bryant, 1940] grew on the exterior of the test pieces and was removed before beetle infestation and analysis. Despite this net loss of N from the system, the percentage amount of N present in the decayed wood is greater than in sound wood because, during decay, major wood components are transformed into water and CO_2 in the vital processes of the fungus at a greater rate than N is removed via the external mycelium. Because of possible variations in N content between sample and sample which cannot entirely be eradicated by any system of matching, the

Table 1. *N contents of oak sapwood before and after decay by* Phellinus cryptarum

(All results expressed as % by weight of oven-dry material.)

Loss in weight due to fungal decay as % of sound wood	N content	
	Found	Calc. as % of sound wood
0·00 (Sound wood)	0·203, 0·197 } 0·200	0·200
17·70	0·230, 0·226, 0·227 } 0·228	0·187
22·65	0·209*	0·162
30·80	0·258, 0·247, 0·246 } 0·250	0·173
36·48	0·225, 0·225 } 0·225	0·143
40·00	0·208, 0·210 } 0·209	0·125
51·20	0·198, 0·193, 0·196 } 0·196	0·096
59·90	0·234, 0·243 } 0·239	0·096
73·30	0·281, 0·284 } 0·283	0·076

* Only one sample available.

results (Table 1) have been examined statistically. The determined average N values, calculated as percentages by weight of sound wood (oven-dry), have been plotted against corresponding values for loss in weight due to decay (Fig. 1) and a regression line determined to express the general relationship between the two quantities. Included in this figure is a probability limits band, within which 95 % of the N content values for all samples of decayed oak sapwood of similar origin would be expected to fall when plotted against the appropriate percentage losses in weight due to decay. Percentage N contents corresponding to each sample of decayed wood actually exposed to beetle attack have been read from the mean line (Fig. 1) to provide the data in Table 2.

The data in Table 2 show that the rate of increase in the N content of decaying oak sapwood is not great and that, until the original wood has lost not less than *ca.* 20 % of its weight by decay, the significance of any increase in the N content of the residue is difficult to establish experimentally when due allowance is made for error in the N determination itself. In view of the nature of the material at his disposal, it is not surprising therefore that Huntgate [1940] concluded that the N content of wood shows little change during decay. It is none the less striking, however, that, in the extreme case, wood which has been so severely decayed as to lose 73·3 % of its original weight (Table 2) may, in the process, have some two-thirds of its N removed via external fungal mycelium and yet leave a residue of N content over 25 % greater than that of the wood itself at the outset of decay. Wood decayed to this extent no doubt contains a relatively high proportion of fungal mycelium.

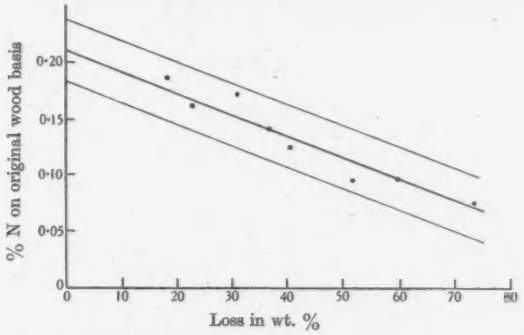

Fig. 1. Relationship between percentage N content and percentage loss in weight due to fungal decay (based on original oven-dry wood).

Table 2. *Calculated mean N content of oak sapwood at various stages of decay by* Phellinus cryptarum

(All results expressed as % by weight of oven-dry material.)

Loss in weight due to fungal decay as % of sound wood	N content: As % of sound wood read from Fig. 1	N content: As % of sample read and calc. from Fig. 1
0·00 (Sound wood)	0·210	0·210
17·70	0·176	0·214
25·62	0·162	0·218
32·80	0·148	0·222
36·48	0·142	0·224
40·00	0·135	0·225
50·60	0·114	0·231
59·90	0·097	0·241
73·30	0·071	0·266

The N economy of the Xestobium *larva reared in decayed wood*

Fisher [1941] concludes from the biological investigation that the effect on larval development of the presence of fungus in the substrate is most pronounced when decay itself is relatively not far advanced, since the rate of decrease in the length of the life cycle was found to diminish considerably when the loss in weight due to decay in the substrate exceeded 45 %. When considered in conjunction with this conclusion the data in Tables 1 and 2 strongly suggest that change in the N content of the substrate is not by itself a determinant of the duration of the larval stage of *Xestobium*. Apart from the fact that up to a loss in weight due to decay of 50 % the N content of the substrate (Table 2) is only about 10 % greater than that of sound wood, the substantial increase in N content of the substrate brought about by advanced decay is not accompanied by a proportionate decrease in the length of the life cycle of the insect. It is therefore of importance to determine as far as possible how the *Xestobium* larva deals with the N which is available to it in substrates of common origin but which differ in the extent of their fungal decomposition. Three sets of material were available for this purpose, and the results of N determinations upon them are given in Table 3.

Table 3. *N contents of three samples of oak sapwood decayed by* Phellinus cryptarum *before attack by* Xestobium rufovillosum *and of the frass produced in them by the larvae*

Loss in weight in sample due to fungal decay as % of sound wood	Average N content (% of oven-dry weight)	
	Decayed wood before insect attack (from Table 1)	Frass
17·9	0·230, 0·226, 0·227 } 0·228	0·235, 0·234 } 0·235
36·48	0·225, 0·225 } 0·225	0·228, 0·223 } 0·225
40	0·208, 0·210 } 0·209	0·221, 0·216, 0·212 } 0·216

When due allowance is made for error in the analytical method used and for possible error due to variations in the distribution of nitrogenous components in the starting material, it must be concluded that for losses in weight due to decay in the substrate up to approximately 40%, the N content of the frass of the *Xestobium* larva is not significantly different from that of the decayed wood in which it bores. The implications of this conclusion are of far-reaching importance, as will now be shown.

Discussion

Throughout these researches data have been obtained for loss in weight due to insect attack and for the weight of frass produced per unit weight of wood disintegrated by boring, so that it is now possible to assess the average N intake of the *Xestobium* larva in the three separate instances dealt with in Table 3 and to relate this intake quantitatively to the total amount of N available in the wood disintegrated (Table 4).

Table 4. *Relationship between N intake and N available to the* Xestobium *larva reared in oak sapwood decayed by* Phellinus cryptarum

(Results, except those in columns (1) and (7), expressed as % by weight (oven-dry) of wood disintegrated by larval boring)

Loss in weight due to fungal decay in substrate as % of sound wood	Loss in weight due to insect attack = abstraction coefficient	Frass produced by larva	N available to larva in wood disintegrated (from Table 1)	N recovered in frass (calc. from Table 3)	N abstracted and retained by larva (by difference)	N abstracted as % of N available per unit of wood disintegrated = N-abstraction coefficient
17·9	47·56	52·44	0·228	0·123	0·105	46·05
36·48	23·93*	76·07*	0·225	0·171	0·054	24·00
40·00	21·15*	78·85*	0·209	0·170	0·039	18·60

* Determined previously by Campbell & Bryant [1940].

The data in Table 4 permit the remarkable conclusion that, irrespective of the extent of decay in the substrate over the range examined, the N-abstraction coefficient of the *Xestobium* larva is not significantly different numerically from the abstraction coefficient for the total food. This means not only that N is removed from the wood digested in the proportion in which it occurs in the substrate, but also that all of the N digested is retained in the larval body

The data in Table 4 actually show that, on the average, 95% of the N in the wood digested is thus retained, but for reasons already given this figure is probably an underestimate. Since, as has already been shown [Campbell & Bryant, 1940], the dietetic value of oak sapwood is not appreciably altered even after extensive fungal decay, it is reasonable to assume that, so far as its N metabolism is concerned, the *Xestobium* larva, to all intents and purposes, always retains all of that element which it absorbs, for if any considerable proportion of metabolized N had been excreted in the frass in the form, for example, of uric acid, amino-acids, or even NH_3, it could not have escaped detection by the methods used. The frass of *Xestobium* must therefore be regarded as consisting essentially of wood which has been comminuted, compressed in part or in whole into pellets in the larval gut, and excreted without having been modified chemically by digestive processes. Wood which is digested, on the other hand, is transformed into soluble products and absorbed virtually *in toto*. Previous claims to have established the composition of the food of *Xestobium* [Campbell, 1929; Ripper, 1930; Norman, 1936] by comparing the composition of frass and sound wood or wood adjacent to larval tunnels are therefore without foundation. In the light of present knowledge, the analytical data recorded in these earlier publications merely give an indication of the changes brought about in the wood by the fungal decay which normally precedes or accompanies beetle attack, and the most accurate estimate of the composition of the larval diet is that afforded by the data of Campbell & Bryant [1940] supplemented by those of the present study. It is realized that this conclusion has an important bearing on the chemical study of the feeding habits of other wood-boring insects.

This demonstration that at least 95%, and probably all, of the N absorbed by the growing *Xestobium* larva is retained in the body, makes it clear that the rate of N intake is one of the principal determinants of the duration of the larval stage in wood.

The experimental data of Fisher [1941], showing the relationship between the length of life cycle and the extent of fungal decay in the substrate, as well as the data of Campbell & Bryant [1940], showing the relationship between the larval abstraction coefficient and the same variable, have been treated statistically to obtain the curves shown in Fig. 2. These two curves are essentially of the same shape, and the common variable may be eliminated by plotting values for length of life cycle (curve A) against corresponding values for abstraction coefficient (curve B). This leads to the curve in Fig. 3 which, because of previous findings (Table 4), illustrates the relationship between length of life cycle on the one hand, and either the total amount of food digested or the amount of N digested per 100 g. of wood disintegrated on the other. It is conceivable that, but for unavoidable errors in the various biological, manipulative and chemical determinations upon which all the foregoing curves are based, the curve in Fig. 3 might have been a straight line. Even as it is, when considered from the standpoint of the N metabolism of *Xestobium*, this curve presents a similar paradox to that encountered in the previous study [Campbell & Bryant, 1940], that as the length of life cycle decreases, so does the amount of N abstracted by the larva from every 100 g. of wood which it comminutes by boring. As has already been shown, this can only be accounted for by an increase in the rate of boring on the part of the larva. The conclusion is thus reached that, in common with the major components [Campbell & Bryant, 1940], the N in decayed as opposed to less decayed or sound wood is made more readily available to the *Xestobium* larva principally on account of the softening effect of fungal decay on the substrate. The larva thereby encounters less resistance to boring and,

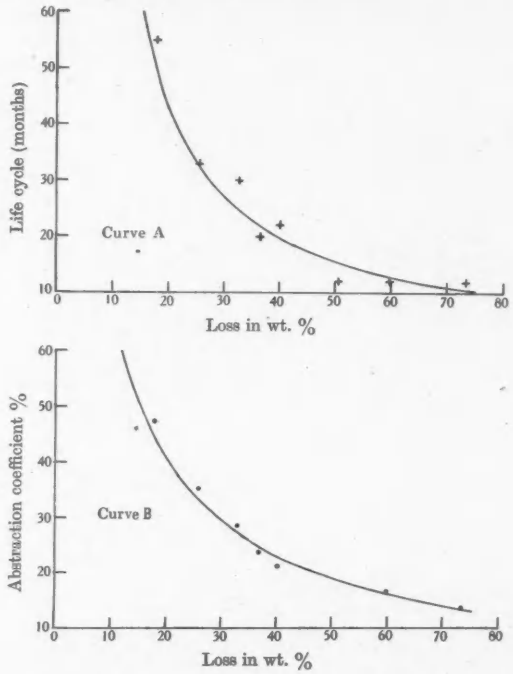

Fig. 2. Curve A. Relationship between duration of life cycle and loss in weight due to fungal decay in the substrate. Curve B. Relationship between larval abstraction coefficient (total food) and loss in weight due to fungal decay in the substrate.

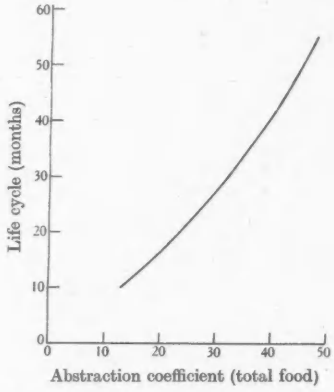

Fig. 3. Relationship between duration of life cycle and (*a*) larval abstraction coefficient (total food), (*b*) larval N-abstraction coefficient.

because it retains substantially all of the N which it digests, it is enabled to complete its development in a shorter time. It follows, of course, that, as the rate of conservation of N increases, so must the rate of conservation of the other elements which are essential to larval growth. For instance, N only represents 1/6·25 of the protein and probably 1/14·5 of the chitin [Armstrong & Armstrong, 1934] synthesized during metabolism. It is calculated from the foregoing data that the larva reared in wood which has lost by decay 73·3 % of its weight must comminute unit weight of wood in approximately 1/12 of the time taken by its counterpart reared in wood decayed to a loss in weight of 17·9 %. Unfortunately an independent method of determining the rate of boring is not as yet available but, for *Xestobium* living either in sound or decayed wood, this is the prime factor which determines the duration of the larval stage and, as shown in previous work [Campbell & Bryant, 1940], when the extent of decay in a suitable substrate is the only variable in the environment of the beetle, rate of boring is determined by the degree of softening thereby induced in the substrate. It is only on a basis of this close integration of effects, which resolve into a reduction in the length of life cycle but emanate from a common cause, that all of the observations, biological and chemical, can be correlated. For instance, there can be little doubt that the shape of curve (Fig. 2), which is characteristic of the relationship between loss in weight due to decay in the substrate on the one hand and each of 'length of life cycle', 'abstraction coefficient (total food)' and 'N-abstraction coefficient' on the other, would also be characteristic of the relationship between loss in weight due to decay (or density) and mechanical strength (or resistance to larval boring) of the substrate. If this be granted, it is readily seen from the slope of the type curve (Fig. 2) why it was observed [Fisher, 1941] that after a loss in weight due to decay in the substrate of about 45 % the rate of decrease in length of life cycle began to decline.

In view of the similarity in the effect of fungal decay on larval growth in both *Hylotrupes* [Becker, 1938] and *Xestobium* [Fisher, 1941], it is reasonable to assume that both insects deal with the N available in the diet in the same way. The results reported by Becker [1938] can best be explained by assuming that when peptone is added to the substrate the *Hylotrupes* larva increases its rate of N intake without having to increase its rate of boring to the extent which it must in decayed wood, where an increase in N intake is only achieved by virtue of an increase in rate of boring.

Summary

1. N estimations have been carried out on a series of samples of oak sapwood decayed to various stages by *Phellinus cryptarum* Karst. The fungus is shown to remove a considerable proportion of the wood N via the mycelium which grows on the exterior of test pieces.

2. After removal of the external mycelium the decayed test pieces were found to be of significantly higher N content than sound wood of similar origin, but the rate of increase in N content of the wood residue during progressive decay was not great. Specimens which had lost approximately 50 % of their original weight by decay might contain, on the average, some 10 % more N than sound wood.

3. The N content of the frass of *Xestobium rufovillosum* De G. reared in oak sapwood decayed by *Phellinus cryptarum* has been compared in three separate instances with that of the original decayed wood, and no significant differences were found between the two quantities on a percentage basis.

4. It is concluded that the growing *Xestobium* larva retains in its body at least 95 %, and probably all, of the N available to it in the wood which it digests and that, because this is so, the rate of intake of N is a determinant of the duration of the larval stage.

5. The N in decayed as opposed to less decayed or sound wood, is made more available to the *Xestobium* larva by the softening effect produced by decay on the substrate. Less resistance is thereby encountered to boring and the duration of the larval stage decreases accordingly.

The work upon which this paper is based was carried out as part of the programme of the Forest Products Research Board, and is communicated by permission of the Department of Scientific and Industrial Research. The N estimations were made by Mr D. F. Packman of the Section of Chemistry and the statistical treatment of the data by Miss C. B. Pettifor of the Section of Timber Mechanics.

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134. THE FERMENTATION PROCESS IN TEA MANUFACTURE. SOME GENERAL CONCLUSIONS

8. THE NATURE OF THE OXIDASE SYSTEM

9. THE RELATION OF TEA-FERMENTATION TO RESPIRATION

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Various aspects of tea-fermentation have been discussed in a series of papers from this laboratory [Roberts & Sarma, 1938; Roberts, 1939, 1, 2; Harrison & Roberts, 1939; Roberts, 1940, 1, 2; Deb & Roberts, 1940; Barua & Roberts, 1940; Roberts & Sarma, 1940; Roberts, 1941]. During the course of this work views have naturally been modified in the light of fresh data, and several statements stand in the literature which the author feels need some amendment; while many of the earlier observations are now seen to have fresh implications. A critical summing up of the work so far published therefore seems to be called for. Additional reason for such a step is furnished by the publication of the results of the Ceylon workers in this field [Lamb & Sreerangachar, 1940]. While agreeing with the Ceylon workers on many points we do not consider that all their experimental methods are completely valid, while some differences that apparently exist may possibly be due to variations in the nature of the material under investigation.

Two main problems seem to be inherent in these investigations, the nature of the oxidase system responsible for tannin oxidation, and the relation of tea-fermentation to normal respiration. There are distinct differences in opinion between Ceylon and Tocklai as to the nature of the oxidase system concerned in tea-fermentation, but evidence will be presented which accounts satisfactorily, if not conclusively, for such differences. As far as the second problem is concerned, in order to avoid misunderstandings our conclusions, considerably strengthened by much unpublished work, will be set out in some detail.

8. THE NATURE OF THE OXIDASE SYSTEM

It was originally claimed [Roberts & Sarma, 1938] that the enzyme responsible for tannin oxidation in tea-fermentation was peroxidase. The main evidence for this consisted in the observation that a strong solution of catalase added to fermenting tea-leaf, damped with an equal quantity of water, strongly depressed the fermentation rate. Balls & Hale [1935] made a similar observation of the effect of catalase on the darkening at the freshly cut surface of the apple. It was suggested by Lamb & Sreerangachar [1940] that this effect in the case of tea

might be due to protease action on oxidizing enzymes, but this criticism does not appear to be valid. Destruction of oxidizing enzymes by protease would have to be very rapid to inhibit the browning of the green tea-leaf after damage, and the author's own experiments [Roberts, 1936] show that xanthine oxidase and other enzymes in the liver extract used preserved their activity for several hours without loss.

I agree with Lamb & Sreerangachar [1940] that peroxidase appears to play no important part in tea-fermentation. This conclusion was arrived at independently before the Ceylon workers produced their evidence to show that leaf could contain both tannin and peroxidase and yet be unable to undergo fermentation.

The role if any of peroxidase in the tea-leaf therefore remains a mystery, nor are we able to account satisfactorily for the inhibition of fermentation by catalase. It is interesting to recall that in dilute suspensions of tea leaf tissue catalase has no inhibitory effect [Roberts, 1940, 2].

The possibility that ascorbic acid oxidase was concerned in tea-fermentation was seriously entertained, but later results showed that the oxidation of ascorbic acid was secondary [Roberts, 1940, 1]. The criticisms of Lamb & Sreerangachar on this point, therefore, only confirm this finding.

Temporary agreement was reached when Lamb & Roberts [1939] suggested that cytochrome oxidase was the enzyme oxidizing tannin during fermentation, but of late our views have again diverged. In the following pages results from both Tocklai and Ceylon will be discussed and an attempt made to account for any outstanding differences.

(1) *Experimental methods*

To a certain extent the inconsistencies between the work on tea-fermentation carried out in the two laboratories must be put down to different methods of attack, and the employment of different experimental techniques. At Tocklai we have carried out our experiments as far as possible under conditions closely resembling those *in vivo*, and have not undertaken any extensive work on the preparation of enzymes from the tea-leaf. Lamb & Sreerangachar have taken the opposite view and have attempted a study of the simpler component reactions of tea-fermentation before embarking on the much more complicated *in vivo* system. It is of course desirable that both aspects of this work should receive attention and it is not surprising that we are unable to reach complete agreement under the circumstances.

As far as techniques are concerned we have largely relied upon manometric methods, paying careful attention to the possible sources of error mentioned by Dixon [1934]. Titrimetric methods have been found to be misleading [Barua & Roberts, 1940] and have not been extensively used. Lamb & Sreerangachar on the other hand have relied almost entirely on the iodine method for polyphenol estimations, although some use has been made of a crude form of respirometer. They admit that the iodine method is not specific for polyphenols, but there is a further drawback in that the condensation of tannins following enzymic oxidation materially affects the titre [Barua & Roberts, 1940].

We have evidence, shortly to be published, which goes to show that whereas oxidation of tannins by the tea oxidase reaches its maximum rate at about 29°, condensation of the tannins accelerates continuously with temperature. At the higher temperatures of fermentation, therefore, extent of condensation is no longer primarily determined by the rate of oxidation and if the iodine titre is affected by condensations the fall in iodine titre is not a true index of tannin

oxidation. It is also likely that the effect of *p*H variance on oxidation and condensation may differ. Before Lamb & Sreerangachar's results on the kinetics of tea oxidase systems can be accepted I therefore feel it is incumbent on these workers to repeat their results using methods less subject to error.

It is quite likely that the errors in the iodine titres due to condensation are small, as the tannin solutions are relatively dilute when compared with fermenting tea-leaf. The concentration of tannin in the juice of leaf withered to 60 % moisture is about 13 %, which is nearly ten times that in the enzyme-substrate mixtures used by Lamb & Sreerangachar. This dilution must result in a very considerable reduction in the extent of condensation. In confirmation of this it has been shown that the Lowenthal permanganate or iodine titres of juice from fermented tea-leaf are considerably reduced if the juice is boiled, but if the juice is diluted 20-fold with boiling water and maintained at the boiling point for 1 hr. the titre is relatively little affected. This experiment indicates a continuance of condensation in the juice at the boiling-point, but not after dilution, where mass-action effects considerably lessen the chances of the necessary molecular collisions.

Results obtained with the crude respirometer by Lamb & Sreerangachar are of doubtful value, but no important findings are based upon its use.

(2) *Soluble and insoluble forms of the tea-oxidase and their kinetics*

Perhaps the most interesting of Lamb & Sreerangachar's findings concern the soluble and insoluble forms of the tea-oxidase. It would appear from the increase in the water-soluble form of the enzyme, after withering the leaf, that the water-soluble enzyme is another form of the insoluble enzyme and not an entirely different substance. It may be recalled that my previous results [Roberts, 1941] show that the normally water-insoluble oxidase becomes soluble as a result of withering. The soluble and insoluble forms have different kinetics, their temperature optima in Ceylon leaf are 49° and 27° respectively, and the optimum *p*H values are 6·8 and 5·4.

The relations of these two forms of the tea-oxidase are somewhat similar to those existing between coenzyme factor (diaphorase) and heart flavoprotein [Corran *et al.* 1939], in which the former is associated with insoluble particles and is inactivated by heat at 55°, whereas the water-soluble heart-flavoprotein must be warmed to 85° for inactivation. As the water-soluble tea oxidase is the more susceptible to inactivation by high concentrations of tannin, its different *p*H optimum may be due to the tannins having a greater inactivating effect at lower *p*H values.

The observation that the water-soluble oxidase is readily inactivated by tannins in high concentration suggests that it plays a comparatively unimportant part in tea-fermentation *in vivo*, for the tannin concentration is high in fermenting leaf. My own observations on the kinetics of tea-fermentation, which follow below, confirm this.

As already shown [Roberts, 1941], the optimum temperature for fermentation is about 29°, in good agreement with the value of 27° for the insoluble oxidase in Ceylon leaf. I have also carried out a series of manometric determinations of the variation of fermentation rate with *p*H. 200 mg. portions of finely ground tea-leaf were suspended in 2 ml. water + 1 ml. buffer (phosphate or phthalate) and the *p*H of the mixture determined with the Beckmann glass electrode *p*H-meter. Autoxidation was apparent at *p*H 6·5 and controls were therefore carried out with boiled tissue suspensions. Fig. 1 shows the relation between the *p*H of the system and the uptakes for the first 20 min. after subtracting the

autoxidation figures. The *p*H optimum is very close to that found by the Ceylon workers for their insoluble oxidase. As both temperature and *p*H optima for the insoluble oxidase agree very closely with the optima found for fermenting tea-leaf there is some foundation for the belief that the water-insoluble oxidase is largely responsible for the oxidation of tannins during fermentation.

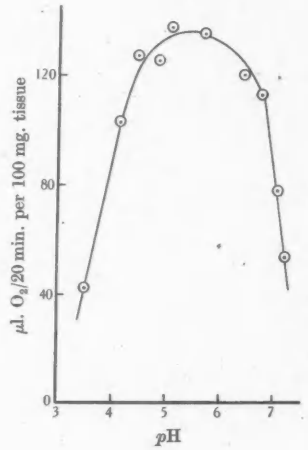

Fig. 1.

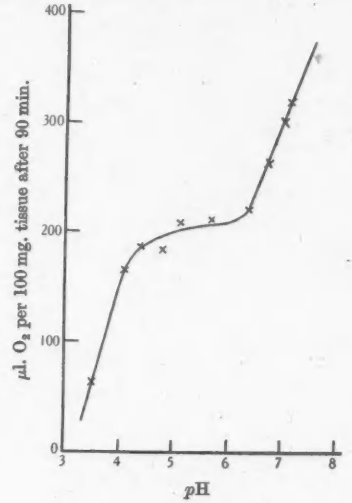

Fig. 2.

The uptakes at *p*H 3·5 slow down considerably after the first 10 min. and the colour of the suspension remains greenish with only a slight tinge of brown. Addition of phosphate buffer *p*H 8, which brings up the *p*H to 5·2, leads only to a slight increase in the rate of uptake, and it is clear that incubation of the tea oxidase at *p*H 3·5 for 20 min. results in a considerable inactivation of the enzyme. On the other hand the enzyme is not destroyed at *p*H 7·2. Adjustment to *p*H 5·4 results in a complete recovery of oxidase activity.

As already stated autoxidation is observed at *p*H values $>6·5$. The end-product of tannin oxidation under such conditions is no longer the *o*-quinone, and the more alkaline the solution the greater becomes the total uptake of O_2. Fig. 2 represents the total uptakes after 90 min. over a range of *p*H from 3·5 to 7·3. At a low *p*H the total uptake is low because of inactivation of the enzyme. From *p*H 4·5 to 6·4 there is little if any effect on the total uptake, but at *p*H $>6·5$, where autoxidation is appreciable, the total uptake increases rapidly with the *p*H.

(3) *Substrate specificity*

Perhaps the greatest differences between our results and those of the Ceylon workers concern the substrate specificity of the enzyme preparations. Lamb & Sreerangachar [1940] deny any oxidation by tea oxidases of quinol and *p*-phenylenediamine, and claim that the observed oxidation of ascorbic acid is due to adsorbed polyphenols. The reasoning in this latter case is not quite convincing, for if, as was shown by Deb & Roberts [1940], catechol is oxidized at

a higher rate than ascorbic acid by the tea oxidase, addition of catechol to enzyme + ascorbic acid must accelerate oxidation of the latter substrate as the *o*-quinone itself can oxidize ascorbic acid.

It is dangerous to draw conclusions from observations on the oxidation of substrates by crude enzyme preparations and the differences between our results here may not be so serious as at first appears. However it is necessary for the cytochrome theory of tea-fermentation for substrates other than *o*-dihydroxyphenols to be oxidized and hence the Ceylon findings are rather disturbing. In view of the probable inactivation by organic solvents of the cytochrome system during preparation, however, these observations may have little significance.

The existence of a cytochrome system in fermented tea-leaf is strongly indicated by the finding that addition of substrates such as ascorbic acid and *p*-phenylenediamine to fermented tea-leaf suspensions, after oxidation of tannins has sunk to low levels (impoverishment), does result in a stimulation of the O_2 uptake. The increase in the oxidation rate shows that these substrates are oxidized directly and not through a tannin or other polyphenol as a carrier. It will be observed that the rate of oxidation of these substrates by 'impoverished' tea-leaf is much lower than that of tea-tannin (Table 1). The results are therefore not completely satisfactory as they might possibly be due to a heavy metal plus protein functioning as a pseudo-oxidase.

Table 1. *O_2 uptakes in μl. for 100 mg. fermented tea-leaf*

	min. ...	15	30	45	60	75
1.	Control	7	14	22	26	—
	Control + P.P.D.	47	88	128	162	—
2.	Control	15	26	—	42	50
	Control + ascorbic acid	23	46	—	83	113

In the second experiment the uptake of O_2 was 17 μl. in the 15 min. immediately preceding addition of ascorbic acid.

(4) *Effect of organic solvents on tea oxidase*

The very considerable extent of inactivation of the oxidase in Assam leaf that attends the washing of tea-leaf tissue with acetone or alcohol first suggested that a cytochrome system was operative in tea-fermentation. Even if the tissue is cooled in ice-salt mixtures during treatment with these solvents inactivation is considerable. The same is true of enzyme preparations obtained by precipitating tea-leaf juice with acetone. The resulting enzyme has only a fraction of the original activity of the juice. For this reason it is unwise to infer the properties of the tea oxidase from those of its preparations obtained by treatment of tea-leaf tissue with acetone or alcohol.

The effects of organic solvents found in our experiments apparently stand in strong contrast to those found by the Ceylon workers. The enzyme preparations obtained in Ceylon all involve the use of alcohol or acetone and are carried out at room temperatures presumably not far from 20°. In view of the strong activities claimed by Lamb & Sreerangachar, who consider that their enzyme preparations contain the greater part of the original oxidase activity of the green leaf, it would appear that the effect of organic solvents is considerably less marked in Ceylon than it is in our case. This may represent a very real difference between Ceylon and Assam materials.

In view of the above possibility it is perhaps unfortunate that the Ceylon experiments appear to be incompletely controlled. A comparison is made between the fermentation of freshly rolled leaf and of an enzyme-substrate mixture diluted with a considerable amount of water or buffer-solution. Such dilution may result in as much as a threefold increase in the rate of O_2 uptake [Roberts, 1940, 2], in which case the above results would show that a very substantial loss of activity attended the acetone treatment.

(5) *Carrier-nature of the tea oxidase*

At various times the non-identity with polyphenol oxidase of the main enzyme concerned in tannin oxidation has been stressed [Roberts, 1940, 1]. Apart from questions of solubility and inactivation by alcohol or acetone, the enzyme appears to bring about oxidation of polyphenols through a carrier. Two independent series of observations have led to this conclusion.

First it was observed that although catechol is oxidized by the oxidase in finely ground tea-leaf at a slower rate than tea-tannin, addition of this substrate to fermenting tea-leaf did not depress the rate of O_2 uptake as it would be expected to if catechol and tannin were in competition for the enzyme. Addition of catechol produced no immediate effect on the uptake of O_2 (Fig. 3), but after a time equivalent to that required for the oxidation of the greater part of the tannin, the rate persisted at a level approximately equal to that of catechol oxidation. This probably means that tannin oxidation proceeds nearly to completion before catechol oxidation begins. Such behaviour is consistent with the oxidation of substances by the oxidized form of a carrier, where oxidation-reduction potentials decide the order of oxidation, but is not generally observed when two substrates are in competition for the same enzyme.

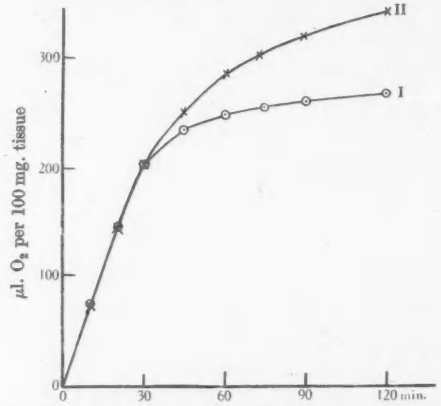

Fig. 3. I, control. II, tea-fermentation plus added catechol ($M/100$).

The second series of observations concerns the variation of the rate of O_2 uptake in fermenting tea-leaf suspension with the degree of dilution of the tissue [Roberts, 1940, 2]. Differences in diffusion rate and variations in substrate concentration cannot explain the increase of Q_{O_2} with the dilution. The best explanation seems to be that at the higher dilutions there is a relatively greater percentage of the carrier in the reduced form, and that with the consequent

increase in true substrate concentration the uptake of O_2 becomes faster. Although the mechanism of this process remains obscure an explanation is to be found in the possible interaction between tannin and cytochrome. This will be considered in a later section.

(6) *Identity of the oxidase in Assam leaf with the cytochrome system*

It is clear from the results quoted by Roberts [1940, 1] and by Deb & Roberts [1940] that the oxidase in tea-leaf responsible for fermentation has many resemblances to the cytochrome system. The tea oxidase however has some properties which appear to distinguish it from the cytochrome oxidase of animal tissues. Thus it undergoes readier peptization and consequently can be freed to a certain extent from insoluble particles. Oxidation of such substrates as quinol and *p*-phenylenediamine is less marked than that of *o*-dihydroxyphenols. This finding may be compared with the statement of Okunuki [1939] that the indophenol (cytochrome) oxidase of lily pollen oxidizes cytochrome-*c* but not quinol. The tea oxidase appears less susceptible to organic solvents, for preparations involving the use of acetone can be employed to effect the oxidation of quinol, *p*-phenylenediamine and the Nadi reagent. The latter oxidation is inhibited by CO but this inhibition is reversed in the light. As such behaviour is typical of cytochrome oxidase it indicates that in plant tissues the enzyme may be only partially inactivated by organic solvents. The tea oxidase is comparatively insensitive to cyanide, 10^{-2} *M* HCN being required for maximum inhibition. Finally the enzyme has a lower optimum temperature and shows its maximum activity at *p*H 5·4. It is inactive at *p*H 3·5 and 7·2.

These very marked differences, however, do not justify us in rejecting the idea that a cytochrome system is responsible for tea-fermentation. Although Bhagvat [1939] has shown the wide distribution of the cytochrome system in plant tissues, there is comparatively little information available as to its properties and we certainly have no right to assume that the properties of the enzyme in the plant will agree closely with those in animal tissues.

Unfortunately the determination of cytochrome and its oxidase in the leaf has special difficulties. Spectrometric investigation is ruled out owing to the masking of the cytochrome spectrum by the chlorophyll. Removal of the chlorophyll involves washing with solvents which largely inactivate the oxidase, and mechanical separation seems of little effect owing to the association of the oxidase activity with chloroplastic material [Roberts, 1941]. Addition of cytochrome-*c* prepared from ox-heart has a slight retarding effect upon the oxidation of tannins by finely ground tea-leaf tissue. This failure to accelerate tannin oxidation however cannot be taken as proof that a cytochrome system is not responsible for tea-fermentation. The possibility arises that the system already contains sufficient cytochrome to bring about the maximum rate of tannin oxidation. It has also to be borne in mind that we are adding a foreign protein to the tea-leaf, and we already have evidence [Roberts & Sarma, 1940] which shows that tea-tannin has a marked inhibitory effect on catalytically active proteins (i.e. enzymes) from other plant sources. As the enzymes of the tea-leaf itself are but little affected by tea-tannin it appears that they possess some type of immunity towards tea-tannin. It is therefore not unlikely that cytochrome-*c* from ox-heart may be inactivated by tea-tannin.

Although we have been unable to produce direct evidence of the presence of cytochrome in the tea-leaf it has been possible to demonstrate its presence in the basal portion of the stem. Thanks are due to Miss K. Bhagvat who undertook a spectroscopic investigation of this tissue for us. An absorption band

corresponding to the *b* band of cytochrome was observed in the green region of the spectrum. On adding succinate and cytochrome-*c* to the finely ground tissue the bands of reduced cytochrome were visible but disappeared on vigorous shaking. Unfortunately the oxidase activity of the tissue was too low to confirm the presence of the cytochrome system manometrically, but the spectroscopic results give clear evidence of the presence of both cytochrome oxidase and succinic dehydrogenase in the tea-plant. Efforts are now being made to observe the spectrum in etiolated tea seedlings.

Perhaps the most serious objection to the cytochrome theory of tea-fermentation is to be found in the very low cyanide sensitivity of the tea-oxidase. Deb & Roberts [1940] have attempted to explain this in terms of competition between cytochrome and other iron compounds for the cyanide, but the observations of Lamb & Sreerangachar [1940], who show that peroxidase activity in fermenting tea-leaf is inhibited by 10^{-5} *M* HCN, render this hypothesis an unlikely one, for the sensitivities of cytochrome oxidase and peroxidase to cyanide are generally observed to be of the same order. It therefore appears that the tea oxidase has a relatively low sensitivity to cyanide. The few data available on other plant tissues [see Deb & Roberts, 1940] show that low sensitivity to cyanide is not confined to the tea oxidase. It seemed desirable to extend these observations, and Table 2 records the inhibition of various plant oxidase systems by HCN. It will be noted that maize embryos retain some oxidase activity in the presence of 10^{-2} *M* HCN. It has been shown by Bhagvat [1939] that germinating maize contains a relatively powerful cytochrome oxidase, so the possibility that plant cytochrome systems have a low cyanide sensitivity becomes much more likely.

The low sensitivity to cyanide may of course be due to impurities associated with the enzyme preparations, although the suppression of peroxidase activity in tea-leaf by 10^{-5} *M* HCN makes this unlikely. Further, Wieland & Sutter [1930] have shown that the potato oxidase (polyphenol oxidase) is 50% inhibited by 5×10^{-4} *M* HCN. The balance of evidence therefore suggests that respiratory oxidases in plant tissues other than polyphenol oxidase and peroxidase require about 10^{-2} *M* HCN for maximum inhibition.

Table 2. *Inhibition of plant oxidases by HCN*

Cyanide conc. ...	*M*/2000	*M*/1000	*M*/500	*M*/100
	%	%	%	%
Tea (fermentation)	45	—	72	85
Tea (respiration)	65	—	73	78
Tobacco (respiration)	—	—	34	58
Maize embryos (respiration)	—	61	—	75
Eugenia balsamea (fermentation) (i.e. tannin oxidation in finely ground leaf tissue suspensions)	—	25	—	77

The percentage inhibition in nearly all the above cases increases with time.

There is no information available as to the *p*H optima of cytochrome systems in plant tissues, although Gräff [1922] has shown that the optimum *p*H range for the Nadi-reaction is on the acid side of neutrality for leaf-tissue, but on the alkaline side for animal tissues. It has also been shown in this laboratory that the oxidation of tannins in a fine mince of *E. balsamea* is optimal at *p*H 5·5. The reversibility of the inhibition at *p*H 7·2 of the tea oxidase suggests that with substrates other than tea-tannin the enzyme might be active at higher *p*H values.

It will be seen that what little information there is on cytochrome systems in the leaf, or on systems which may possibly be identified with the cytochrome system, does indicate that there are some differences in its properties when compared with those of the animal cytochrome system. More information is needed on plant oxidase systems, but in the meantime it is claimed that the properties of the tea oxidase are such as to justify its tentative identification with a cytochrome oxidase.

In conclusion the assumption that cytochrome acts as a carrier in the oxidation processes in the tea-leaf enables a new explanation of the concentration effect, described by Roberts [1940, 2], to be made. It has already been suggested that tea-tannin may combine with the cytochrome-*c* of ox-heart and so render it ineffective as a carrier. Combination of tea-tannin with the cytochrome of the tea-leaf is also a possibility, but is not likely to be so complete, by analogy with the effect of tannins on plant oxidases. The amount of free, uncombined cytochrome in suspensions of finely ground tea-leaf may therefore be determined by mass action effects, and in the more dilute suspensions its concentration would be expected to be relatively higher. On this hypothesis, as dilution increases the amount of free cytochrome per unit quantity of tissue also increases, and the initial rate of O_2 consumption may also be expected to increase.

(7) *The oxidase system in Ceylon leaf*

Lamb & Sreerangachar [1940] have amassed a considerable body of evidence which seems at first sight to make it unlikely that cytochrome oxidase is responsible for tannin oxidation in their leaf.

Some of their observations, notably the failure of cytochrome-*c* from ox-heart to promote more rapid tannin oxidation, and the low sensitivity of tannin oxidation to cyanide, have been confirmed, but it is not considered that these facts are incompatible with the functioning of a cytochrome system in tea-fermentation, as was shown in the preceding section.

It must be remembered that all the Ceylon workers' results were obtained with enzyme preparations in which the tissue had been treated with acetone or alcohol at a temperature of probably about 20°. This treatment would have led to a marked decrease of cytochrome oxidase activity, despite the apparent resistance of the plant oxidase to destruction by organic solvents. The failure to obtain evidence for the oxidation of quinol and *p*-phenylenediamine by their enzyme preparations is therefore not surprising. Using the sensitive manometric method we obtain only weak activity towards these substrates with our preparations, where the treatments with alcohol or acetone have been carried out at 0°.

I have already cast doubt on the claim made by Lamb & Sreerangachar that their enzyme preparations contain practically the whole of the original oxidase activity of the tissue. However, it does not appear unlikely that the enzymes of the Ceylon leaf are rather more resistant to organic solvents than those in Assam leaf, and from the figures quoted by Lamb & Sreerangachar there is reason to believe that catechol may be oxidized by the Ceylon preparations at a higher rate than tannin, although this requires further confirmation. With our preparations the reverse is the case.

This could be taken to imply that different oxidase systems are concerned in the tea-leaf from Ceylon and Assam respectively, but I am reluctant to advance so extreme a hypothesis.

On the other hand we know from the work of Boswell & Whiting [1938] and Steward *et al.* [1940] that other respiratory enzymes in addition to the polyphenol

oxidase are concerned in the respiration of the potato tuber, and in many animal tissues it is impossible to make cytochrome oxidase responsible for the total consumption of O_2. Now the conditions under which tea is grown vary considerably, and in the high altitudes in Ceylon and the plains of Assam not only are there taxonomic differences in the bushes to start with, but there are also considerable differences in environment, soils and the climatic conditions. In Assam the bushes are pruned annually and for three months in the year there is a dormant period in the growth. In Ceylon, on the other hand, growth is fairly continuous throughout the year and the bushes are pruned at three- or four-year intervals. Such differences may be expected to have their effect on the relative proportions of oxidizing enzymes in the leaf, and it is possible that the cytochrome oxidase plays a rather less dominant role in Ceylon leaf than it does under our conditions. The existence of a bush, whose leaves fail to undergo the colour changes characteristic of fermentation unless a tea-oxidase from other bushes is added to them, shows that the oxidase responsible for tannin oxidation is not the only one concerned in the respiration of the tea-leaf. This observation, in my opinion, provides convincing evidence of the existence of more than one oxidase system in the tea-leaf.

The suggestions made are tentative only, and the question of the true nature of the tea oxidase is left an open one. It may be thought that the evidence for a difference between Assam and Ceylon leaves is rather tenuous. My intention is merely to point out that such differences may possibly be expected and that there are already indications which point in this direction. It is clear that much further work both on the tea oxidase or oxidases and on plant oxidases in general is needed. One additional point however may be made. It has recently been shown by Tuzimura *et al.* [1940] that riboflavin occurs in Japanese green teas. This is a strong point in favour of the presence of cytochrome in the tea-leaf and it suggests that the normal cytochrome-flavin-phosphopyridine chain is concerned in the transfer of O_2 to the respiratory substrates.

Discussion

From consideration of the results obtained in this laboratory only, the available evidence goes to show that the oxidase responsible for tea-fermentation is probably to be identified with a cytochrome system. Differences in the properties of this enzyme from those of the cytochrome oxidase of animal tissues exist, but it must be emphasized that little is known to date of cytochrome oxidase in plant tissues, and almost nothing of the oxidase in the leaf.

The results obtained in Ceylon do not differ seriously from our own except in the matter of substrate specificity. The data also suggest that catechol is oxidized faster than tannin by the enzyme in Ceylon leaf, and that organic solvents have a less pronounced effect on the activity of the oxidase than in our experiments. These differences if they are confirmed would suggest that at least two oxidases are concerned in the oxidation of tea-tannin, and that the relative proportions of these oxidases may be affected by differences in cultural and climatic conditions such as exist between the plains of Assam and the hills of Ceylon. Although this possibility must be borne in mind the evidence is by no means conclusive and the observed differences may be a consequence of the use of different techniques in the estimation of enzymic activity.

SUMMARY

The activity of the oxidase in fermenting tea-leaf is maximal at *p*H 5·4. The enzyme is destroyed at *p*H 3·5, but although activity at *p*H 7·2 is low, readjustment to *p*H 5·4 restores the full activity.

The properties of the tea oxidase are compared with those of cytochrome oxidase and reasons are advanced for supposing that cytochrome systems in plant tissues differ in several important respects from those in animal tissues.

There may be more than one oxidase in the tea-leaf, and it is suggested that variations in the relative amounts of these oxidases under varying conditions of culture may account for some of the possible differences in properties found in Assam and Ceylon leaf.

9. THE RELATION OF TEA-FERMENTATION TO RESPIRATION

BEFORE discussing the mechanism of the tea-fermentation process and its relation to respiration it is necessary to deal with the extent of oxidation of the tannins that takes place as the result of fermentation, and to consider the extent of mechanical damage to the tissue.

Statements have been made in earlier papers [Roberts, 1939, 2; Harrison & Roberts, 1939] as to the amount of O_2 consumed in tannin oxidation when fermentation is complete. These may now be amplified.

The total amount of O_2 consumed in fermentation may be determined manometrically; after 60 min. shaking the rate of uptake is low and the total uptake after 90 min. is very little lower than the maximum attainable. Total uptakes have been determined in 52 different experiments, each in triplicate or quadruplicate, and the average value from all these results is 11·85 μl./mg. dry weight with a standard error of 0·11 μl. only. Some of this O_2 is used up in carbohydrate oxidation and assuming the oxidation to follow the conventional course, $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$, the amount of O_2 used up in oxidizing tannins may be obtained by multiplying the total uptake by $(1-x)$ where x is the R.Q. From 22 separate experiments the R.Q., when 200 mg. of finely ground tea-leaf are suspended in 3 ml. water at 36°, is $0{\cdot}367 \pm 0{\cdot}007$. The average consumption of O_2 in tannin oxidation is therefore $11{\cdot}85 \times 0{\cdot}633$ which amounts to 7·5 μl./mg. dry weight.

Several thousand tannin contents of green tea-leaf have been determined at Tocklai in the last 20 years and the average figure 22 % or 0·22 mg./mg. dry weight has an entirely negligible standard error. Assuming a molecular weight of 300 for tea-tannin [Harrison & Roberts, 1939] this is equivalent to an O_2 consumption of 0·91 atom per mol. In view of the assumptions made we can say with some confidence that 1 atom of O_2 is consumed for every molecule of tea-tannin.

In these manometric experiments the leaf is ground to a fine paste. The point has been raised that we cannot be certain that a considerable proportion of the tissue has not escaped damage. The CO_2 produced could then be considered as due to the respiration of intact cells, in which case it must be assumed that only 63·3 % of the cells have been ruptured. Our calculation now leads to the conclusion that 1·44 atoms of O_2 are consumed per mol. of tannin. This is highly improbable and could only be accounted for if our estimate of the mol. weight of tannin were seriously at fault.

The supposition is made unlikely when we consider the extent of tannin oxidation in fermenting tea-leaf under factory conditions. With the twisted leaf obtained after mechanical rolling in the factory, fermentation slows down considerably after 2–3 hr. although enzymic activity is still high. This suggests completion of oxidation of the substrate. If the fermented leaf be finely ground to a paste and then suspended in water there is a small further uptake of O_2 amounting to 0·6 to 3·0 μl. O_2/mg. dry weight. Assuming a round figure of 12 μl. O_2/mg. dry weight for complete fermentation this is equivalent to an uptake of 9·0–11·4 μl. O_2 or 75–95 % completion of tannin oxidation in the fermented leaf. The same answer may be obtained in an entirely different way. Extraction of the tissue with hot water for 1 hr. results in almost quantitative removal of the unoxidized tannins, and if such an extract be added to finely ground fresh tea-leaf tissue, the O_2 consumed after 90 min. by the tissue is increased by an amount equivalent to the unoxidized tannin in this extract. It is always found that the observed increase agrees closely with the residual uptakes obtained by a fine grinding of the fermented leaf.

This close agreement not only proves that the leaf is sufficiently damaged in the mechanical rolling to realize approximate completion of the fermentation process, i.e. from 75 to 95 % oxidation of the tannins, but also shows that the fine grinding results in the oxidation of all tannins extractable by boiling water. It also follows that the damage inflicted on the tissue need not be sufficient to rupture each individual cell. Microscopic examination of well fermented tea-leaf reveals that the majority of the outer cell walls are intact. It is therefore probable that the effect of the twisting of the leaf in inducing fermentation is upon the internal arrangement of the cell.

The persistence of carbohydrate oxidation and production of CO_2 during fermentation might be considered to be due to normal respiration, but below we offer clear evidence to show that damage to the tissue, sufficient to bring about complete oxidation of the tannins, is also sufficient to cause complete suppression of respiratory activity. The carbohydrate oxidation observed in tea-fermentation is to be considered a secondary oxidation by the tannin oxidation products.

After completion of tannin oxidation in tea-fermentation, CO_2 production falls to a very low level, and in non-tanniferous leaves such as *Tropaeolum* [Roberts & Sarma, 1940] fine grinding of the tissue leads to almost complete inhibition of O_2 uptake. The most convincing evidence for the suppression of normal respiration by mechanical damage however is provided by measurements of the anaerobic fermentation [Deb & Roberts, 1940]. Production of CO_2 under anaerobic conditions can readily be demonstrated for the whole tea-leaf, but in the ground up paste used in manometric experiments no CO_2 production can be detected when the suspension is shaken in an atmosphere free from O_2. Similarly, with well-twisted leaf such as is obtained by mechanical rolling in the tea factory, up to 98 % inhibition of anaerobic fermentation is observed. On the other hand, leaf passed through a coarse meat chopper retains about 30 % of its anaerobic fermentative powers and many green, undamaged pieces of tissue can be detected in the coarse mince which do not undergo the characteristic tea-fermentation without further bruising.

The secondary nature of the oxidation of carbohydrates in tea-fermentation is clear from the strict dependence of the rate of CO_2 production on the rate of tannin oxidation and from the suppression of carbohydrate oxidation by the addition of ascorbic acid, which reduces *o*-quinones as soon as they are formed.

It is perhaps desirable to present a balance sheet for the oxidation of carbohydrate during fermentation which shows that our assumption of the origin of CO_2 during this process is a valid one. To quote a typical experiment, the reducing sugars expressed as glucose fell from 2·32 to 1·13 % as a result of a 3-hr. fermentation. (The original withered leaf was free of starch and hydrolysable non-reducing sugars.) The complete oxidation of this quantity of glucose would be equivalent to an output of 8·89 μl. CO_2/mg. dry weight. Figures for the CO_2 output of fermenting leaf are available only for suspensions of finely ground tissue in water, but the amount of CO_2 evolved where the volume of the suspending fluid is zero can be obtained by extrapolation [Roberts, 1940, 2] and amounts to 8·90 μl. CO_2/mg. dry weight. The excellent agreement is probably fortuitous. However, in several experiments the mean fall in reducing sugars as a result of fermentation amounts to 1·20 % of the total dry weight, so that we are certainly justified in concluding that the CO_2 produced in fermentation comes largely from carbohydrate breakdown.

Recapitulating the above arguments the following experimental facts emerge.

(1) In the course of tea-fermentation nearly the whole of the tannins is oxidized.

(2) The mechanical damage necessary to cause complete oxidation of the tannins results in a complete suppression of normal respiration.

(3) Such carbohydrate oxidation as occurs is a secondary oxidation with the *o*-quinone of tea-tannin as the H-acceptor.

(4) After correcting for the amount of O_2 consumed in oxidizing carbohydrates, the uptake of O_2 corresponds to a consumption of 1 atom per molecule of tannin.

The *o*-quinone of tea-tannin if not reduced again is removed irreversibly from the system by condensation to form bodies with more marked tannin properties [Harrison & Roberts, 1939]. The main chemical changes in tea-fermentation may therefore be summarized:

(1) Tea-catechins + $O_2 \xrightarrow{\text{Oxidase}}$ *o*-quinones

(2) *o*-Quinone + glucose $\xrightarrow{\text{dehydrogenases}}$ tea-catechins + CO_2

(3) *o*-Quinones $\longrightarrow$ condensed tannins

or more shortly

(4) Catechin $\underset{\text{Dehydrogenase}}{\overset{\text{Oxidase}}{\rightleftharpoons}}$ *o*-quinone $\rightarrow$ condensed tannin,

and the process will continue until the whole of the tea-tannin has been converted into the oxidized-condensation products found in 'black' tea.

How are we to account for this effect of extensive mechanical damage on the respiratory mechanism of the plant cell? Reaction (4) emphasizes the fact that both oxidase and dehydrogenase activities are relatively unimpaired. Partial inactivation of dehydrogenases cannot account for the phenomena observed, as anaerobic fermentation in the bruised tea-leaf and respiratory activity in crushed non-tanniferous leaves would otherwise be noticed. It therefore appears that carriers between oxidase and dehydrogenase are affected by mechanical damage. As the phenomenon of pigmentation after damage is observed in systems containing the polyphenol oxidase, which do not require the assistance of a flavin in the oxidation of substrates, it is probable that some form of coenzyme inactivation is responsible, this being the carrier common to polyphenol and cytochrome oxidase systems.

There is a difficulty in accepting this mechanism at first sight. The complete suppression of anaerobic fermentation by extensive damage requires complete destruction, or rather dispersal, of coenzymes, whereas the relation of carbohydrate oxidation to *o*-quinone concentration requires some coenzyme activity. It was suggested by Roberts & Sarma [1940] that the *o*-quinone of tea-tannin might function as a H-acceptor for dehydrogenase action without the need for a coenzyme. However, it has now been shown that addition of catechol or pyrogallol to fermenting tea-leaf materially increases the CO_2 production (Fig. 4). As Dixon & Zerfas [1940] have shown that *o*-quinone itself will not function as a H-acceptor in the absence of a coenzyme it is evident that the concentration of coenzymes in fermenting tea-leaf is sufficient to ensure secondary oxidation of carbohydrates by the *o*-quinones of tea-catechins.

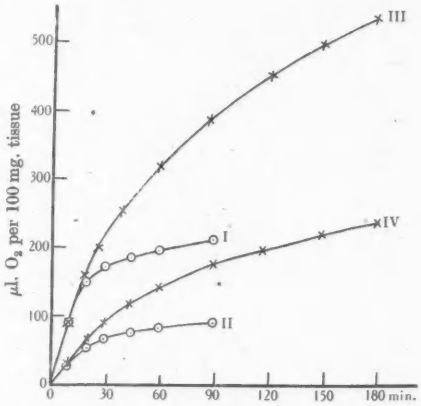

Fig. 4. I, O_2 uptake, control fermentation. II, CO_2 output, control fermentation. III, O_2 uptake with added catechol ($M/100$). IV, CO_2 output with added catechol.

It is now possible to account for this apparent inconsistency. In the intact cell oxidation of substrates probably takes place at interfaces consisting of a complex mosaic of protein and phosphatide molecules. Adsorption of coenzymes at specific points adjacent to both oxidase and dehydrogenase is necessary before substrate oxidation can occur. The organization of such an active surface can be destroyed by a shearing stress quite inadequate to rupture the comparatively robust cell wall. In this connexion it may be remembered that Phillis & Mason [1937] have shown that the result of a slight shearing stress on leaf tissue has a very marked effect on the composition of the continuous phase in the cytoplasm, and hence on the permeability to solutes of the cytoplasm. With the organization destroyed the chances of oxidase, carrier, substrate and dehydrogenase all being in sufficiently close contact to effect oxidation of the substrate are very considerably reduced and respiratory activity in consequence is largely inhibited.

The fermentation process however does not need this organization. After mechanical damage to the cell the tannin is no longer confined to the vacuole but penetrates into the cytoplasm so that it is in intimate contact with the oxidase system, and *o*-quinone formation will in consequence be rapid. The coenzymes like the tannins are probably in homogeneous solution and must be largely

oxidized by the *o*-quinones and will be able to function as H-acceptors in substrate oxidation by the dehydrogenases. The presence in the system of a relatively high concentration of *o*-quinones provides the necessary link between oxidase and dehydrogenase which was lost when the organization of the cell was destroyed. Under these conditions some dehydrogenase activity will be apparent until the *o*-quinones have been irreversibly lost by condensation.

That *o*-quinone concentration is correlated with the rate of carbohydrate oxidation is shown by the variation of the R.Q. with the dilution of tissue suspensions [Roberts, 1940, 2]. As the dilution increases, the concentration of *o*-quinone and hence also of the oxidized form of the coenzymes decreases; so that carbohydrate oxidation becomes relatively less rapid.

The effect of withering on the R.Q. *of fermenting tea-leaf*

Measurements of the R.Q. of both fresh and withered tea-leaf have shown that the R.Q. of the undamaged tissue is 1·0 within the limits of experimental error. The R.Q. for a suspension of finely ground tissue equivalent to 15 mg. dry weight per ml. is 0·37 for both fresh and withered leaf. The variation of the fermentation rate with dilution [Roberts, 1940, 2] is also the same for both fresh and withered leaf.

These experimental facts are re-emphasized in view of the statements made by the Ceylon workers. It is as easy to obtain a good fermentation with unwithered leaf as it is with withered leaf, in fact in some parts of North-east India where conditions for satisfactory withering are rarely obtained the withering process is omitted. It is certainly easier to produce the necessary damage to withered leaf, but fresh leaf despite its turgor can be dealt with satisfactorily and the fermentation times required are if anything somewhat shorter than after withering.

It is considered that withering has an effect, although a small one, on the rate of fermentation [Roberts, 1941], but none of our results indicates the profound effects hinted at by Lamb & Sreerangachar [1940]. The results of Evans [1928] quoted by these authors cannot be accepted. The method by which these figures were obtained is not disclosed, but it is well known [Dixon, 1934] that measurements of gaseous interchanges are liable to very serious errors if gaseous diffusion is not very carefully controlled.

SUMMARY

The effect of mechanical damage to the tea-leaf is to suppress normal respiration and anaerobic fermentation almost completely. This damage need not involve mechanical rupture of each individual cell. With a suitable shearing stress the organization within the cell is so destroyed that the coenzymes can no longer function as links between oxidase and dehydrogenase. A second consequence of the destruction of the organization of the cell is the penetration of the cytoplasm by the tannins.

The tannins are now oxidized directly by the oxidase system. Oxidation to the *o*-quinone stage is complete and condensation following this oxidation converts the tannins into a series of brownish-red pigments. When in the *o*-quinone stage the tannins can effect secondary oxidation of carbohydrates with the aid of the coenzymes dispersed through the tissue. During fermentation carbohydrate oxidation is only observed while *o*-quinones are being formed, and is stopped by removal of the *o*-quinones by condensation or reduction with ascorbic acid.

The author wishes to thank Mr P. H. Carpenter, the Director, for his continued interest in this work and the Indian Tea Association for permission to publish these results. He also wishes to express his gratitude to Miss K. Bhagvat, with whom he has discussed this work, for her valuable criticisms and many useful suggestions.

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135. THE RATE OF REMOVAL OF AMINES FROM THE BLOOD

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Previous work on the amine oxidase has suggested that this enzyme is concerned primarily in the detoxication of amines produced by bacterial action in the gut [Richter, 1938; Richter & Tingey, 1939]. Amines are readily destroyed by preparations of the enzyme *in vitro*; but no information has hitherto been available as to the rate at which amines are detoxicated by this enzyme and removed from the blood *in vivo*.

The rate of detoxication of amines in the body is of particular significance in pharmacological work, in which it is desirable to know for how long after administration amines are still active in the blood. Further information as to the rate of inactivation of amines *in vivo* also appeared desirable in view of the suggestion that certain pathological conditions may be due to autointoxication by amines [Alvarez, 1924; Looney, 1924; Stewart, 1929; Quastel, 1937]. Amines are known to be produced not only by the putrefactive bacteria occurring normally in the intestine, but also by pathogenic strains, such as those of *Bact. typhosum* and *C. welchii*, which may invade the tissues, and amines have been isolated from pus [Kaunitz & Wacek, 1935; Lachampt, 1938; Gale, 1940]. Several of the amines produced by bacteria, such as putrescine, cadaverine and the lower aliphatic amines, are relatively non-toxic; but β-phenylethylamine, tyramine and the higher aliphatic amines show marked sympathomimetic activity and are toxic even in very small quantities. In the normal healthy individual these amines are readily destroyed by the amine oxidase in the liver, intestine and other tissues [Bhagvat *et al.* 1939]; but it has been suggested that in pathological conditions the detoxication may be deficient, giving rise to an abnormally high level of amines in the blood [Quastel, 1937].

Quastel & Wheatley [1933] have obtained evidence of the way in which amines might act in exerting their toxic action; but there is no evidence at present available as to the actual concentration of these amines in the blood in normal or pathological conditions. It appeared that for the further investigation of this problem there are required (*a*) a highly sensitive method for estimating traces of amines in the blood, and (*b*) a method of testing the efficiency with which amines are detoxicated *in vivo*. An attempt has therefore been made to devise such methods and to use them for obtaining further data on normal subjects.

The estimation of amines in blood

The usual methods for estimating 'amino-nitrogen', such as those of Folin or of Van Slyke, are not sufficiently sensitive for estimating the traces of amines that might be present in blood, and as they apply to amino-acids and amino-compounds in general a preliminary separation of the amines from the other amino-derivatives would be necessary. The colorimetric method using the amine

picrate [Richter, 1938] is sufficiently sensitive and is specific for amines or amino-compounds that are extracted from aqueous solution by organic solvents. Preliminary experiments showed that specimens of normal blood frequently contain a substance that gave a strong amine reaction when tested by this method. The substance was non-volatile and was not decomposed by nitrous acid; it was identified as a phospholipin, probably lecithin, which reacts by virtue of the quaternary amino-group in the choline residue. Added lecithin behaved in a similar manner. The method was therefore modified in order to avoid interference from lecithin.

Principle of method. The amines are extracted from whole blood by shaking with light petroleum and an excess of K_2CO_3, which liberates the free amines and at the same time exerts a salting out action. The loss of amine through combination with traces of aldehydes in the blood to form Schiff's bases, which may become significant at the lower amine concentrations, is reduced by adding a small amount of tyramine, which is not extracted. The amines are separated from phospholipins by taking them into a small volume of *N* H_2SO_4 saturated with NaBr; the phospholipins remain in the light petroleum solution. The amines are then taken back into light petroleum and picric acid is added to form the picrates, which give a strong yellow colour in chloroform solution.

Procedure. The blood was collected in a tube containing potassium oxalate as anticoagulant and 10 ml. blood were transferred to a 30 ml. centrifuge tube and stirred with about 5 mg. solid tyramine hydrochloride. Light petroleum (10 ml. of B.P. 80–100°) and saturated K_2CO_3 solution (5 ml.) were added and the tube was shaken vigorously for 3 min. The emulsion was broken down by alternately centrifuging and stirring with a thick glass rod: 7 ml. of the light petroleum layer were then pipetted into a 10 ml. centrifuge tube containing 0·5 ml. of a solution of *N* H_2SO_4 which had been saturated with NaBr. The mixture was shaken for 4 min., centrifuged and the light petroleum layer discarded. The acid was washed by adding 3 ml. fresh light petroleum, which, after shaking for 3 min. and centrifuging, was again discarded. The amines were now taken back into organic solvent by adding 2·4 ml. light petroleum and 0·2 ml. 40 % KOH and shaking for 6 min. After centrifuging, the light petroleum layer was transferred to a clean, dry test tube; it was mixed with 2·4 ml. chloroform and the colour was developed by adding 0·1 ml. 2 % picric acid in chloroform. The colour was then compared with a series of standard solutions prepared by adding known amounts of β-phenylethylamine or *iso*amylamine to samples of blood (up to 50 μg./ml.) and treating them in the same manner. For some purposes the direct comparison by eye in test-tubes of uniform bore was sufficiently accurate, but greater accuracy was obtained by using a photoelectric absorptiometer with Ilford spectrum violet filters. A calibration curve giving amine concentration × absorptiometer reading was first prepared with the standard solutions; the amine concentration in solutions of unknown strength could then be obtained from this curve. A blank using 10 ml. distilled water was repeated with each set of estimations.

Discussion of method. It is essential that the light petroleum and chloroform used should be free from amines or other compounds giving a colour with picric acid, so that a good blank is obtained. Different samples of light petroleum and chloroform were found to vary greatly in this respect: one specimen of technical light petroleum gave a better blank than the 'A.R.' The solvents must therefore be tested and purified if necessary by extracting with acid before use. In view of the sensitivity of the method to traces of acids or alkalis it was found necessary to clean all the apparatus used with cleaning mixture and to pay special attention

to the thorough rinsing and drying of the test tubes and pipettes. The tubes were kept stoppered as much as possible to prevent evaporation of the petroleum. Clean corks covered with cellophane were generally used for shaking. The partition of the amines between the aqueous and organic phases is affected considerably by the proteins present in blood; amine solutions in blood and not aqueous solutions were therefore used in preparing the standards for colorimetric comparison. Five specimens of normal blood which had been taken for transfusion and which were used in the preliminary experiments showed no trace of amine when tested by this method: one specimen of ox blood and one of guinea-pig blood were also found to be free from amines.

The method is specially adapted to the estimation of amines such as β-phenylethylamine or the higher aliphatic amines which are produced by the putrefactive bacteria and which are readily soluble in organic solvents. The sensitivity differs for different amines according to their solubilities and molecular weights; the following figures give an approximate estimate of the colour intensities obtained with aqueous solutions containing 20 μg./ml. of a number of different amines, expressed in terms of the concentration of β-phenylethylamine (μg./ml.) which gave the same intensity of colour:

Trimethylamine	16	Tryptamine	3
*iso*Amylamine	20	Tyramine	0
Putrescine	0	*sec*Butylamine	20
Mescaline	4	Ephedrine	8
Methylamine	0	Creatinine	0

The method of taking the amine into a small volume of acid enabled a concentration of the amine to be effected. The sensitivity of the method for a given amine depended on the volumes taken for the different extractions. The volumes given were chosen arbitrarily as being convenient, but the sensitivity was increased by using 1 ml. of light petroleum instead of 2·4 ml. for the final extraction and adding 1 ml. of chloroform. Under these conditions an addition of 1 μg./ml. of β-phenylethylamine to normal blood could be readily detected.

The rate of detoxication of amines in vivo

Some indication of the rate of detoxication of β-phenylethylamine was obtained in experiments reported previously [Richter, 1938] in which the amine was taken by mouth and the rate of excretion of phenylacetic acid in the urine was measured. The amount of acid excreted in 2¼ hr. corresponded to 40 % and in 4½ hr. 62 % of the theoretical amount expected from the 300 mg. amine which were taken. This showed that the rate of detoxication is rapid; but the rate of absorption of the amine in the gut and the rate of coupling of the phenylacetic acid with glutamic acid (it is excreted as phenylacetylglutamine) come in as unknown factors which would have to be determined before the true rate of detoxication could be deduced from experiments of this kind.

Blood-pressure test

A further indication of the rate of detoxication was obtained by measuring the duration of the blood-pressure rise after β-phenylethylamine had been administered intravenously. The systolic and diastolic blood pressures were measured by the auscultatory method with a sphygmomanometer applied to the brachial artery. The subject for the test was a patient (62 kg.) with hysterical symptoms, but in whom a careful examination had failed to show any physical

abnormalities. The solution for injection contained β-phenylethylamine hydrochloride (5 % base) in distilled water and it was sterilized by keeping at 100° for 1 hr. Blood pressures taken before and after the intravenous injection of 1·5 ml. (75 mg. base) are recorded in Fig. 1.

The systolic blood pressure rose sharply to 220 mm. Hg after the injection, but was back to normal again within $2\frac{3}{4}$ min. This suggested that the rate of detoxication of β-phenylethylamine might be very rapid; it would be of the order of 26 mg./kg. body weight/hr., if the amine were all destroyed when the blood pressure returned to normal.

The blood pressure test is very easy to carry out and might perhaps be used clinically as an indication of amine oxidase function; but it is doubtful whether one is justified in assuming that the return of the blood pressure to normal coincides with the complete detoxication of the amine. It appeared that a more

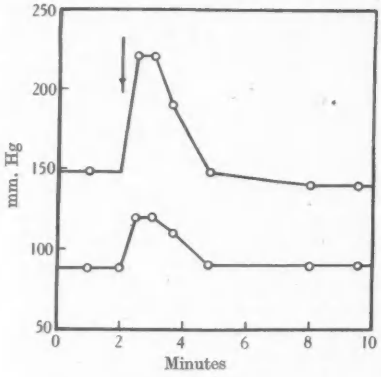

Fig. 1

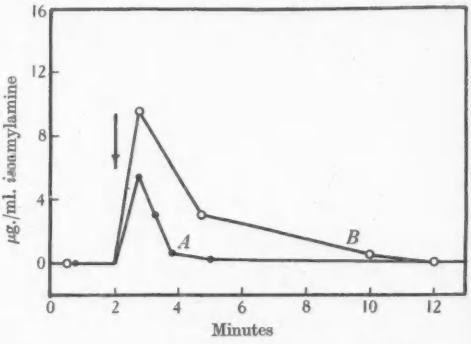

Fig. 2

Fig. 1. Blood pressure, systolic above and diastolic below, after the intravenous injection of 75 mg. β-phenylethylamine at the time marked by the arrow. Subject (62 kg.).

Fig. 2. Concentration of *iso*amylamine in the blood after the intravenous injection of 500 mg. *iso*amylamine at the time marked by the arrow in subjects *A* (60 kg.) and *B* (66 kg.).

reliable indication of the rate of detoxication would be obtained by estimating directly the amount of amine remaining in the blood at different times after an intravenous injection of an amine.

Amine removal test. β-Phenylethylamine is too toxic for it to be administered in sufficiently large doses to be estimated conveniently in the blood. *iso*Amylamine, which is relatively non-toxic, was found to be more suitable for this purpose. A solution of *iso*amylamine hydrochloride containing 12·5 % of the base was sterilized for intravenous injection by heating at 100° for 1 hr. In preliminary trials with gradually increasing doses the dose was raised to 4 ml. of the 12·5 % solution (500 mg. base) for subjects of about 60 kg. and with this dose the reaction was never unpleasantly severe.

Procedure. In carrying out this test 4 ml. 12·5 % *iso*amylamine were introduced into a 5 ml. syringe and several clean, dry 10 ml. syringes were kept ready for withdrawing samples of blood. With the subject in the reclining position 10 ml. of blood were taken from the left arm. The amine solution was injected into the median cubital vein of the right arm and at intervals of 40 sec., 100 sec.,

3 min. and 10 min.; after the injection, further 10 ml. samples of blood were withdrawn from the left arm. The blood was collected in tubes containing potassium oxalate and the amount of *iso*amylamine remaining in the blood was estimated as above.

Five tests were carried out as described on subjects who had been treated for mild nervous disorders but who were physically normal so far as could be ascertained at the time of the tests: the results of two such tests are shown in Fig. 2. The time required for the amine to be removed from the blood ranged from about 2 to 8 min.; this would indicate a rate of removal of the order of 60–250 mg. *iso*amylamine/kg. body weight/hr.

An indication of the fate of the amine was obtained with one subject in whom it was observed that the blood smelt strongly of *iso*amyl alcohol in the samples taken 100 sec. or more after the injection. The removal of amine from the blood in the first few seconds immediately following the injection must be due in part to the diffusion of the amine out of the blood and into the tissues, but the final removal of all the amine from the blood must be ascribed to an irreversible detoxication. The amine is first oxidized and deaminated by the amine oxidase and the resulting aldehyde is then oxidized further to the acid or converted into a mixture of the corresponding alcohol and acid by the aldehyde oxidase and aldehyde mutase.

The efficiency of the amine-detoxicating mechanism is inversely proportional to the area under the amine removal curve (Fig. 2). This gives a method of measuring the amine oxidase function, which might be expressed as

$$\frac{\text{mg. amine administered}}{\text{area} \times \text{kg. body weight}}.$$

The amine oxidase function was of the order of 1 to 2 in the normal subjects who have been examined. In view of the sensitivity of the method of estimating blood amines, extreme accuracy cannot be claimed for this method of measuring the amine oxidase function; but the method would appear to be adequate for demonstrating any gross defect in the amine-detoxicating mechanism.

Discussion

The idea that toxic amines might play a part in disease derives mainly from the work of Barger and Dale, who first examined the physiological properties of a large series of amines and demonstrated that they are produced in putrefactive processes. Autointoxication by amines has been suggested to explain the hyperglycaemia and other symptoms observed in intestinal obstruction. It has also long been known that the symptoms observed clinically in certain types of nervous disease can be reproduced artificially by administering amines such as mescaline or other β-phenylethylamine derivatives, and this has led to the view that toxic amines might play a special part in these disorders [Richter & Lee, 1941].

Apart from a few attempts to estimate blood histamine and adrenaline, no figures have hitherto been available for the normal level of amines in the blood. There has also been no information as to the efficiency of the normal mechanism for detoxicating amines *in vivo*. The results obtained by the methods now described indicate that human blood normally contains less than 1 part per million of amines of the β-phenylethylamine or *iso*amylamine types, and that the body is normally capable of detoxicating about 4–15 g. *iso*amylamine per hour. This is much greater than the rate at which amines are likely to be formed

under normal conditions by bacterial action in the gut and suggests that the body has a considerable capacity in reserve for detoxicating amines.

In a study of the reactions of 5 men who voluntarily induced constipation lasting for 90 hr., Donaldson [1922] observed the symptoms of coated tongue, foul breath, hyperglycaemia, impaired appetite, mental sluggishness, loss of attention, restlessness, irritability, depression, poor sleep, increased reaction time and headache. Donaldson concluded that these symptoms could not be attributed to toxic substances in the blood, since the symptoms were promptly removed by giving enemata, while he assumed that detoxication must be a relatively slow process. This assumption is now shown to be incorrect as far as amines are concerned, for injected amines are completely removed from the blood within a few minutes.

The method of estimating blood amines which has now been described applies to β-phenylethylamine and the higher aliphatic amines, but not to tyramine, histamine or amines insoluble in organic solvents. However, the conditions (acid medium) which favour the production of β-phenylethylamine and the aliphatic amines also favour the production of tyramine and histamine, which are likely to be present under conditions in which amines of the former type are found.

The amine oxidase function test indicates the efficiency of the detoxication of all the amines oxidized by this system; these include β-phenylethylamine, the aliphatic amines, tyramine and tryptamine, but not histamine. It is suggested that the amine oxidase function test and the other tests described should be used for the clinical investigation of conditions in which intoxication by amines is suspected.

Summary

1. A sensitive method is described for estimating amines in the blood. The method is capable of detecting 1 part per million of β-phenylethylamine or *iso*amylamine.

2. Specimens of normal ox blood, guinea-pig blood and human blood contained less than 1 part per million of amines when tested by this method.

3. The rate of detoxication of β-phenylethylamine administered intravenously in man was estimated from the time taken by the blood pressure to return to normal to be of the order of 26 mg./kg. body weight/hr.

4. The rate of detoxication of *iso*amylamine administered intravenously in man was estimated from the rate of removal from the blood to be of the order of 60–250 mg./kg. body weight/hr.

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136. NOTES ON THE ESTIMATION OF THE MEMBERS OF THE SULPHANILAMIDE GROUP, WITH SPECIAL REFERENCE TO SULPHATHIAZOLE

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THE control of the commonly prescribed members of the sulphanilamide group of preparations is usually carried out, at the present time, by one of the methods or modifications of the methods devised by Marshall. These methods are based on the diazotization of the free or liberated amino group of the drug and subsequent coupling with a substance which will give a suitable azo-dye.

In Marshall's [1937] first and perhaps least known method, the diazo-compound was coupled with dimethyl-α-naphthylamine in alcohol; in his second method [Bratton & Marshall, 1939] the diazo complex is coupled with *N*-(1-naphthyl)-ethylenediamine dihydrochloride in aqueous solution. Making use of his own modification of the first method, the difference lying mainly in the substitution of water for alcohol as solvent, Osterheld [1940] of the 'Ciba' Research Laboratories found unexpected difficulties in the estimation of 2-(*p*-aminobenzenesulphamido)thiazole (i.e. sulphathiazole), especially when dealing with urine. We, however, found that if we adhered strictly to the conditions laid down in Marshall's first method, the estimation of sulphathiazole presented no difficulty other than that inherent in the method, namely the final filtration of the solution to remove precipitated fats, lipoids etc.

Osterheld advises the use of acetyl H acid (1-acetamino-8-hydroxy-3:6-naphthalenedisulphonic acid) as a coupling reagent, since the red azo-dye formed is soluble in water, but as he points out, it is necessary to use a very pure specimen of the acetyl H acid; such commercial specimens as we have been able to obtain give dark brown solutions which have a large blank value in the colorimetric estimation. The pure acetyl H acid has the advantage that it couples to give a dye soluble in all concentrations in sodium acetate solution, and moreover it is indifferent to moderate excesses of nitrite since it does not couple on itself. The *N*-(1-naphthyl)-ethylenediamine dihydrochloride used by Marshall in his second method gives a somewhat more intense colour than that obtained with acetyl H acid with all members of the group, but in the case of sulphathiazole the azo-dye is much less soluble and tends to separate in flakes even in dilute solution, and the tendency is much exaggerated in the case of urine. These difficulties mentioned by Osterheld concerning the insolubility of the dye do not appear to such a great extent when sulphathiazole is being estimated in blood filtrates by Marshall's second method, provided that the proper precautions are taken, and on consideration we noted that the main difference between the procedures for blood and urine was the use of trichloroacetic acid in the former; when this reagent was added to the urine, clear solutions were obtained with the highest concentrations usable with either an absorptiometer or a colorimeter, though a certain amount of precipitation did occur when the solutions were allowed to stand for more than half an hour.

In this estimation it is more accurate and convenient to make use of the Spekker photoelectric absorptiometer, or some such instrument, for the comparison of the coloured solutions, rather than the visual colorimeter, since the variation of colour with concentration only obeys Beer's law within quite narrow limits of concentration. Using an absorptiometer, the graphs obtained of concentration against drum reading (a logarithmic scale) produce a picture of which the illustration given below is typical. A glance at the curve shows that, using a

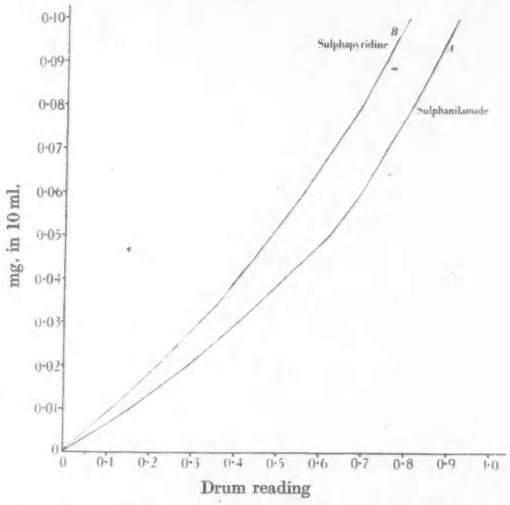

Fig. 1. Colour-production/concentration curves for sulphanilamide (*A*) and sulphapyridine (*B*), with the Spekker photoelectric absorptiometer.

colorimeter, the use of a single standard of say 5 mg./100 ml. for comparison would lead to considerable error when the unknown is of any higher concentration than the standard, since above that level the curve does not obey Beer's law, the depth of colour produced dropping steadily with the rise in concentration. In comparing say a 10 mg./100 ml. concentration against a 5 mg./100 ml. standard in a colorimeter, the error may well be as high as 15–20 %.

In compiling the curves, three separate sets of series of standard solutions were taken, but owing to the accuracy of the instrument this is not usually necessary for calibration, provided that sufficient care is taken; our variations were rarely more than 2 %. Owing, however, to the idiosyncrasies of each instrument, it is pointless to give tables of figures, since each instrument must be individually calibrated for the estimation of each substance.

The curve for sulphanilamide is similar to, but different in degree from, that of sulphapyridine, but either may be used for the other with no great error, provided that the concentration read from the graph is corrected for the difference in molecular weight. The curve for sulphathiazole is almost identical with that for sulphapyridine, probably because of the similarity in the molecular weights.

Method

For both the blood and urine estimations two curves should be prepared, one for the free drug, and the other for the hydrolysed material, as the change in *p*H due to added HCl, necessary for the hydrolysis, appears to damp down the colour production.

Reagents.

1. 15% trichloroacetic acid.
2. 0·1% sodium nitrite solution, freshly prepared.
3. 0·5% ammonium sulphamate solution.
4. 0·05% saponin solution.
5. 4*N* HCl.
6. 0·1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in water. This solution should be kept in a brown bottle.
7. A stock solution of sulphanilamide, sulphapyridine or sulphathiazole containing 200 mg./l. for preparation of the calibration curves. 5 ml. of this solution are mixed with 18 ml. of 15% trichloroacetic acid and the solution is diluted to 100 ml.; 10 standards, containing 1 to 10 ml. of this solution, are each made up to 10 ml. with a solution of 18 ml. of the 15% trichloroacetic acid per 100 ml., and are treated as described below.

Procedure for blood. 2 ml. of oxalated blood are measured into a flask, diluted with 30 ml. of saponin solution and, after 1–2 min., precipitated with 8 ml. of trichloroacetic acid. After standing for 5 min. the liquid is centrifuged.

The free sulphanilamide in the supernatant liquid is determined as follows: to 10 ml. of the clear liquid is added 1 ml. of sodium nitrite solution, and after 3 min. 1 ml. of ammonium sulphamate is added to destroy excess nitrite; after a further 3 min. 1 ml. of the *N*-(1-naphthyl)-ethylenediamine dihydrochloride is added and the colour produced is either measured in the absorptiometer or against an appropriate standard in a colorimeter.

To determine the total sulphanilamide, 10 ml. of the supernatant liquid are treated with 0·5 ml. of 4*N* HCl and the mixture is heated in a loosely stoppered tube in a boiling water bath for 1 hr. After cooling the volume is made up to 10 ml. and the solution is treated as above.

Procedure for urine. The urine is diluted to contain 1 to 2 g. of sulphanilamide per 100 ml. and 50 ml. of the diluted urine with 5 ml. HCl are made up to 100 ml.; 10 ml. of this dilution are treated as for blood filtrates to determine the free drug, and 10 ml. are heated without further addition for the determination of the total sulphanilamide.

In the case of sulphathiazole, 18 ml. of 15% trichloroacetic acid are added during the second dilution of the urine, and no trouble should be experienced due to precipitation provided that the coloured solutions are not allowed to stand for more than 20–30 min.

A large number of experiments were also made using a great variety of coupling reagents, in the hope of finding an azo-dye of sufficient colour intensity for use in a micro method, but so far we have found nothing to compare with the *N*-(1-naphthyl)-ethylenediamine dihydrochloride.

SUMMARY

1. The difficulties encountered by Osterheld in the estimation of sulphathiazole in urine have been overcome.

2. The colour-production/concentration curves on an absorptiometer have clearly shown that more than one standard is essential if the comparison is made with a colorimeter.

For some of the reagents we are indebted to Dr K. H. Saunders of the I.C.I. Ltd., and to Dr A. Batley of Kodak Ltd.

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137. THE RETENTION AND ELIMINATION OF FLUORINE IN BONES

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The occurrence and role of fluorine at toxic and non-toxic levels in animal nutrition is of increasing interest. Much of the older quantitative work is of little value because only recently have reliable methods been devised for the estimation of traces of this element.

The present work aimed at investigating (1) the F content of human bones at different ages, under conditions in which there were no obvious signs of fluorosis: (2) the uptake and elimination of F from the bones of rats which had received toxic amounts of NaF.

Assuming that minute non-toxic quantities of F are taken in, it would be reasonable to expect that the F content of calcified tissues would rise with increasing age, for several reasons. First, natural deposits of calcium phosphate have a special affinity for F, as a result of which rock phosphates contain significant amounts of this element, which are also to be found in fossil teeth and bones. This last fact was probably responsible for the idea that F is a constant and considerable constituent of dental tissues, in particular the enamel in which it occurs as fluorapatite. Secondly, the F ion is toxic; it forms insoluble Ca salts and so presumably one method of detoxication might be to store it as apatite in the teeth and bones.

From the work reviewed by Roholm [1937] the conclusion may be drawn that owing to faulty methods of determination, there are insufficient data on which to give a final answer as to the relation between age and F content in the absence of signs of F intoxication. Roholm [1937] made a set of determinations on adult human bones and found an average of 0·093 % F (lowest 0·059 %, highest 0·21 %). The average F content of land animal bones, including man, has also been determined recently by Klement [1938], who gave the value of 0·05 % of the inorganic substance of bone and suggested that there is probably an increase with age. This was also the opinion of Sharpless & McCollum [1933] working on rats. Marcovitch & Stanley [1938], who determined the F content of the whole eviscerated bodies of rats at birth, at 29 and at 90 days, came to the conclusion that the F content of the whole body decreased considerably with increasing age, but this has no direct bearing on the present work, which deals only with bones.

In the past much divergence of opinion has existed with regard to the role of F and its occurrence in the tissues because of the difficulty of estimating it unless careful attention is given to details. We have used the colorimetric method in which the F bleaches a zirconium-alizarin lake and have followed the details and design of apparatus for the distillation of the F as HF, employed at the laboratory of the Ministry of Agriculture and Fisheries. We are greatly indebted to Dr H. H. Green of that laboratory for his advice in this matter.

1. *The fluorine content of human rib bones at different ages*

The specimens of human bones used in this investigation (25 samples) were obtained from two of the large London hospitals. Rib bones were chosen because they were easy to obtain. The bones were collected from persons who had been resident in London, which up to the present has not been considered a fluorosis area. Certain sources of London water contain up to 0·5 p.p.m. but the tap water would contain less than this.

After post mortem examination the bones were placed in alcohol for a few days, after which they were treated for several days with acetone to free them from fat and water. They could then easily be ground to a fine powder on a rotating circular file. This powder was again extracted with acetone, passed through a fine wire mesh and dried. For the spectroscopic examination the powder was used straight away. Details of the spectrographic method have already been published [Lowater & Murray, 1937]. We took the opportunity of analysing the bone ash for Ca, P and Mg. For these determinations the powders were ashed directly and the usual standard methods employed. For the chemical determination of F the powders were ashed with magnesia ashing mixture, a suspension of 1 % MgO in 7 % Mg acetate, account being taken of the F in all reagents by blank determinations.

Spectrographic analyses. The spectrographic analyses were carried out before the chemical analyses so that a qualitative comparison could be made of the F contents of the bones. The bone specimens were handed over as a series of numbered samples. When the spectrograms were complete, the specimens were arranged in the order of increasing F content, as judged from the spectrograms. This order proved to be in general the order of the age of the subjects. The results are set out in Table 1 and plotted in Fig. 1.

Table 1

Cause of death	Age years	Samples in the order of increasing F content judged from spectrograms
Asphyxia	1	1
Hydrocephalus	½	2
Full-term foetus (stillborn)	0	3
Pneumonia	2	3
Cerebral haemorrhage	6	4
Uraemia	11½	5
Mastoid	14	6
Lymphatic leukaemia	15	7
Mitral stenosis	16	8
Accident	22	9
Acute uraemia, nephritis	14	10
Cardiac failure, nephritis	30	11
Cardiac failure	30	12
Staphylococcal septicaemia	21	13
Died in labour	25	14
Suppurative cholangitis and jaundice	37	15
Bacterial endocarditis	27	16
Bacterial endocarditis	29	17
Cause not noted	32	18
Carcinoma of stomach	59	19
Fractured vertebrae	60	20
Pulmonary embolism and tuberculosis	68	21
Heart failure	37	23

Having obtained a relationship between age and F content of the bones, we carried out a set of chemical determinations on the bones, the results of which are given in Table 2.

When these results are plotted side by side with the spectrographic results they show, on the whole, an increase of F content with age. From simple statistical tests it appears that with the exception of the value found at 22 years and the second of the values found at 37 years the results are not inconsistent

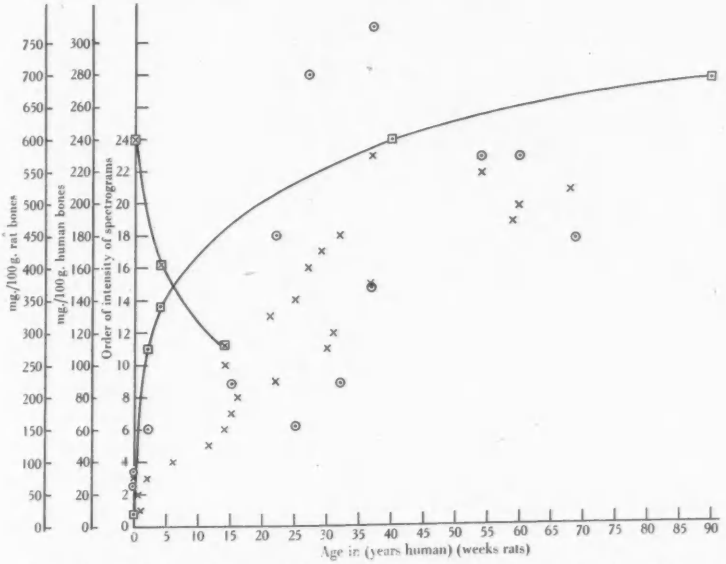

Fig. 1. Showing (1) Order of F content of human bones in relation to age taken from the spectrograms; denoted ×. (2) F content of human bones in relation to age; denoted ⊙. (3) Uptake of F by rat bones on a diet containing 0·05% NaF; denoted ⊡. (4) Elimination of F from rat bones; denoted ⊠.

Table 2. *Analyses on human bones at different ages*

Age years	Ca %	P %	Ca/P	Mg %	Ash %	F % in fat-free bone
	in ash of bones					
0	—	—	—	—	—	0·024
0	—	—	—	—	—	0·033
2	—	—	—	—	52·1	0·06
6	40·13	17·72	2·26	0·72	—	—
15	—	—	—	—	50·5	0·088
22	40·24	17·18	2·34	0·88	45·3	0·18
25	40·02	17·30	2·31	0·48	—	0·061
27	—	—	—	—	47·4	0·28
29	40·33	17·61	2·27	0·78	—	—
32	40·65	17·77	2·29	0·78	—	—
32	—	—	—	—	45·8	0·089
33	40·11	16·87	2·37	0·52	—	—
37	40·46	17·22	2·35	0·62	45·1	0·31
37	—	—	—	—	53·4	0·15
54	—	—	—	—	—	0·23
59	38·21	17·05	2·24	0·65	—	—
59	39·53	17·28	2·29	0·64	48·5	0·09
60	40·66	16·93	2·42	0·50	48·6	0·23
68	40·52	17·28	2·35	0·88	47·3	0·18

with a law that the F increases by 0·002 % for each year. The chemical results also show how remarkably constant the composition of the bone ash is. In two cases the Ca differed more than usual from the mean value. The Mg figures show fluctuations which appear to be without significance. This was somewhat surprising, since we had been led to expect, from the work of Day *et al.* [1935] on rats, that the Mg content would show a progressive increase with age. These rats were all given the same diet, whereas that of the human subjects must have varied widely.

2. *The uptake and elimination of fluorine by the bones of rats during and after fluorine feeding*

Rats were fed from the age of 6 weeks on the diet used in previous work [Murray, 1936]. The NaF, when included, was given as a constant proportion of the diet which was moistened by the addition of 1 ml. of 0·05 % NaF per g. of diet, with a maximum intake of 15 g. of diet per day. The actual diet itself contained 0·00047 % or 4·7 p.p.m. F. At different ages control rats and F-fed rats were killed and the long bones analysed for F. The bones of the F-fed rats were unusually white, thickened and very brittle. Table 3 gives the F content of the long bones of the rats at different ages. A set of determinations was also made of the F content of the bones of two groups of rats which had been on the diet for about 36 weeks but which had been taken off the diet for different periods before being killed. Table 3 also shows the fall in F content, i.e. the elimination from the bones in these two groups, which were of different stocks.

Table 3. A. *Uptake of F by the long bones of rats on a diet containing 0·05 % NaF*

Age weeks	Time on NaF weeks	% F in fat-free bones
6	0	0·019
90	0	0·028
98	0	0·040
8	2	0·273
10	4	0·343
14	8	0·428
16	10	0·644
46	40	0·600
80	74	0·617
90	84	0·705

B. *Elimination of F from the bones after withdrawal of the NaF from the diet*

Series (*a*): rats on the NaF diet 40 weeks

Age weeks	Time from the withdrawal of NaF till death weeks	% F in fat-free bones
46	0	0·600
50	4	0·407
60	14	0·287

Series (*b*): rats on the NaF diet 32 weeks

38	0	0·943
42	4	0·673
50	12	0·302

It appears that the uptake of F is rapid at first and then becomes more gradual, in fact the results when plotted fit a logarithmic curve. This might merely be due to the growth rate, but in view of the elimination rate, is not

necessarily so related. The highest concentration found was 0·943 %. In human cases of fluorosis resulting from the intake of cryolite dust, the highest concentration reported has been 1·3 % [Roholm, 1937]. Elimination seems to follow a course similar to that of the uptake. Unfortunately we have not carried out this process over a long enough period to secure complete elimination, but the results suggest that in human cases of fluorosis a fair degree of recovery might be expected when there was no further exposure to F. A similar experiment has been reported on a cow which had been exposed to a F-containing pasture [Bosworth & Green, 1941]. The animal was removed and grazed on a F-free pasture; the F content of the rib bones fell rapidly at first, but a good deal was still retained and being excreted at a time when the cow no longer showed clinical signs of fluorosis.

Summary

1. The fluorine contents of human bones of different ages have been compared spectrographically and determined chemically. There is in general a rise of fluorine content with increase of age. The lowest value was 0·02 % and the highest recorded here, with no evidence of fluorosis, was 0·3 %.

2. Rats fed small amounts of NaF take up fluorine in the bones, the concentration of fluorine in relation to age following a logarithmic curve.

3. After withdrawal of the fluorine from the diet the elimination from the bones is the inverse of the uptake.

We wish to record our thanks to the Medical Research Council for an expenses grant and for a personal grant to G. E. G. during the course of this work. Our thanks are also due to Mr J. H. Awbery of the National Physical Laboratory for the statistical examination of the results on the human bones.

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138. THE INFLUENCE OF INTERMITTENT CONSUMPTION OF VITAMIN C ON THE DEVELOPMENT OF SCURVY

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It is customary to speak of the daily requirement of a dietetic constituent. This, of course, does not mean that in order to avoid catastrophic results the dose in question has to be consumed with regularity every day, for it is evident that, owing to the uncertainties associated with the struggle for existence, no species could have survived under such conditions. It is comprehensible that the daily dose need not be regular in the case of constituents which are capable of storage in the body, even though our knowledge of all the stages in the mechanism involved in the utilization of food by the body is imperfect. The problem, however, becomes more arresting when consideration is given to those dietetic substances which appear, according to our present knowledge, to be incapable of storage, at least to any appreciable extent. Vitamin C is a case in point. The writer has shown [Zilva, 1936] that vitamin C is not stored by the growing guinea-pig for reserve purposes. Thus he found that fully saturated and unsaturated animals alike developed scurvy on a scorbutic diet; they succumbed to it under similar conditions, the time taken for the first manifestation and for the fatal issue of the disease being of the same order in each case. This observation called for further investigation, particularly of the behaviour of these animals when the minimum dose instead of being administered daily was offered intermittently in amounts which increased in proportion to the number of days between the doses, so that the same total amount was given in each case over the whole period of experiment. This communication deals with a few simple experiments which throw some light on the subject.

It is obvious that in order to obtain the necessary information, it was essential that the minimum dose, or a dose approximating to it, should be used as a standard. The minimum dose does not lend itself to precise definition even in the case of the guinea-pig, in which, unlike the human being, a good opportunity of obtaining more exact results is afforded owing to the possibility of controlled experimentation involving the examination and analysis of tissues at will. In the majority of growing guinea-pigs on a scorbutic diet a daily dose of 1 mg. is sufficient to prevent the appearance of macroscopic signs of scurvy. About double that dose is required to obviate microscopic changes in the pulp of their teeth [Goettsch, 1928], and a daily dose of 5–8 mg. per day is necessary to bring about the deposition of a discernible trace of ascorbic acid in their selective organs [Zilva, 1936].

In this investigation daily doses of 2 and 5 mg. were adopted as standards. The requisite multiple of the former dose was administered to a number of groups of growing guinea-pigs on a scorbutic diet either daily, every other day, every third day or weekly. The equivalent of seven daily doses of the latter

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standard was similarly given once a week (i.e. a dose of 35 mg.). One set of these groups was fully saturated in respect of vitamin C at the beginning of the experiment. Another set was previously maintained for 8 days on a scorbutic diet and these animals thus became unsaturated, containing at the most only traces of ascorbic acid in their tissues. The duration of the experiment was 90 days, at the end of which period the animals were killed by chloroform and autopsied. The constitution of the basal diet and the conditions under which the animals were maintained were the same as those usually employed in this laboratory, details of which have already been given by the writer in previous communications [cf. Kellie & Zilva, 1941].

The results are represented graphically in Figs. 1 and 2. It is immediately evident that the responses of the guinea-pigs in each set of the experiment, namely those which were saturated and those which were unsaturated at the beginning of the tests, were almost identical. The behaviour of the guinea-pigs receiving the 2 mg. dose daily, or its multiple every other day or every third day respectively, was in all cases similar and uneventful; full protection from macroscopic scurvy and good growth were achieved in all these groups. Those, however, which received 14 mg. of ascorbic acid once a week showed definitely poorer growth, and in some of the animals very slight signs of scurvy were observed at the post mortem examination. A striking feature in all these groups was the similarity of the reaction of the saturated and the unsaturated animals. A saturated guinea-pig of about 300 g. contains 15–20 mg. of ascorbic acid in its body (unpublished results), and it would have been expected that if this quantity acted as a store its action would have been reflected at least at some stage of the experiment. This, however, does not appear to have been the case, and the lack of any significant differences between the two groups therefore lends further support to the writer's view that the body ascorbic acid of the saturated animal does not act in any marked degree as a reserve for the vitamin C requirement. When offered 35 mg. once a week, i.e. the equivalent of 5 mg. instead of 2 mg. per day, both the saturated and unsaturated guinea-pigs grew well and were fully protected from macroscopic signs of scurvy. It is, however, of importance to bear in mind that 5 mg. per day approximates to the 'minimum' dose, since it represents the lower limit of a range of daily doses (5–8 mg.) which are just capable of depositing minute traces of ascorbic acid in the tissues.

A clearer interpretation of the above results can be obtained if they are considered in terms of the human being. It must be realized in instituting such a comparison that it can only be of a tentative nature, but everything points to the fact that some conclusions can thus be drawn with a fair degree of probability. A saturated guinea-pig when placed on a scorbutic diet loses its body ascorbic acid rapidly. The greater part of it disappears from the tissues in 3–5 days, and after 8 days the vitamin is in most cases hardly demonstrable by the available methods. Well-declared signs of scurvy are usually found *post mortem* in well nourished animals after 21–24 days, although this condition may sometimes be demonstrated even after 14 days. Death from the disease ensues after 30–35 days. In the case of the human being on a scorbutic diet the disease runs a similar course. Quantitative data are for obvious reasons not as easily obtained as with guinea-pigs. The body ascorbic acid probably disappears after about 2 months. Crandon *et al.* [1940] found that the plasma ascorbic acid level fell rapidly and reached zero after 41 days. In Rietschel & Mensching's [1939] experiment it fell to 0·17 mg./100 ml. of serum in 58 days. A determination carried out after 100 days gave a zero value. No agreement is found in the literature as to the time taken for the first appearance of the scorbutic symptoms.

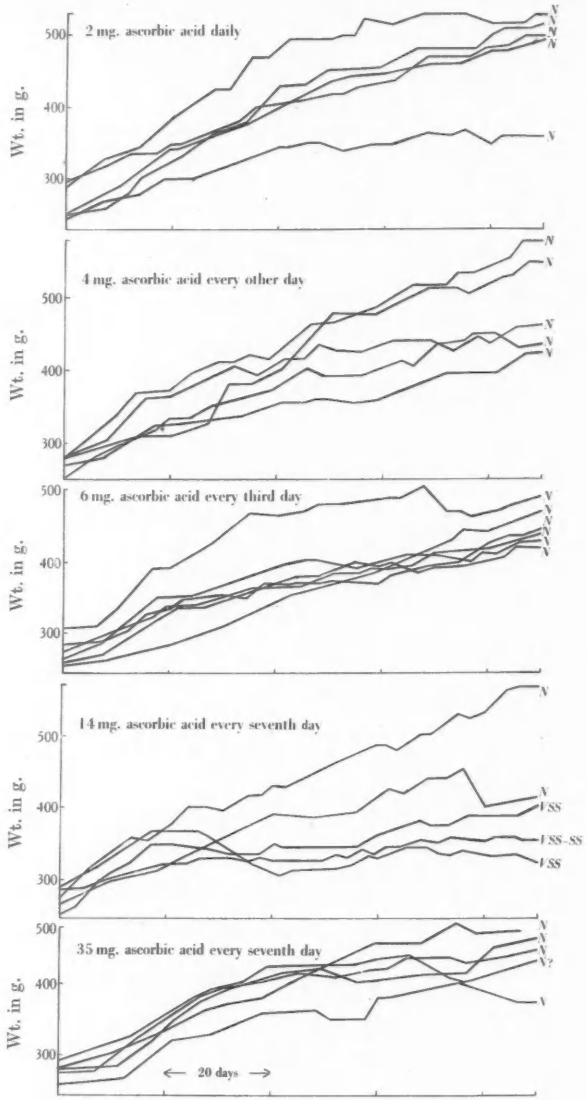

Fig. 1. Saturated at beginning of experiment.

N = Normal.
N? = Very small haemorrhage in right knee joint suspected to be due to injury.
VSS = Very slight scurvy.
SS = Slight scurvy.

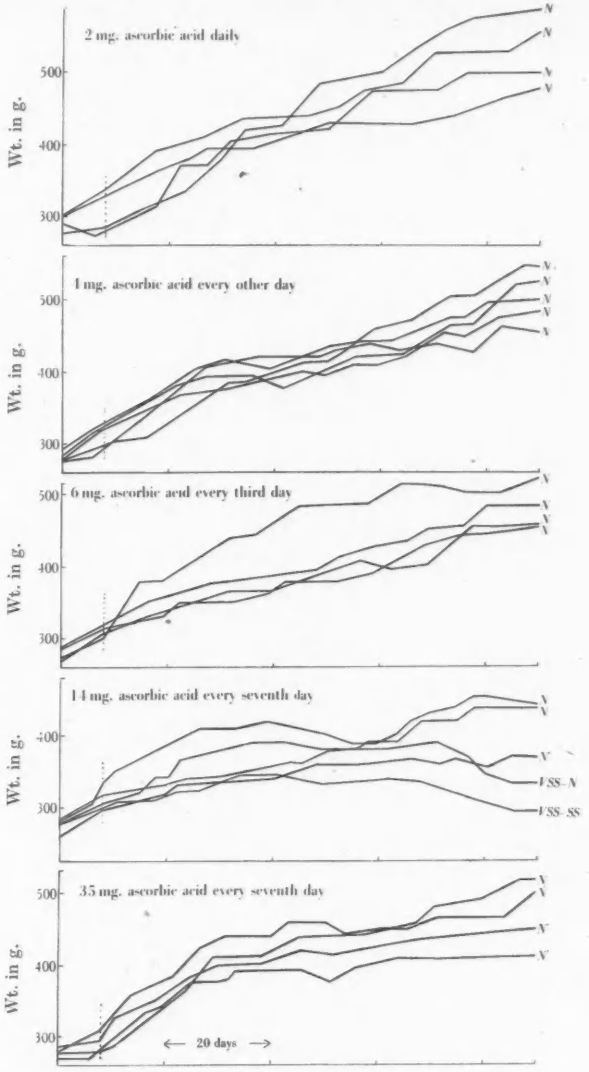

Fig. 2. Unsaturated at the beginning of experiment.

| = Commencement of administration of dose.
N = Normal.
VSS = Very slight scurvy.
SS = Slight scurvy.

This is not surprising, alike owing to individual variations, to differences in the conditions of existence of the subjects influencing the precipitation of the symptoms, and above all to the uncertainty of the absolute scorbutic character of the diet. We have, however, the data of a recent and exceptionally well controlled experiment available as a standard [Crandon *et al.* 1940]. Crandon placed himself (for a period of 6 months) on a diet entirely deficient in vitamin C, but supplemented by all the other known dietary constituents. The first clinical signs of abnormality, hyperkeratotic papules, appeared after 132 days, but 161 days elapsed before the first specific signs of scurvy, the perifollicular haemorrhages or petechiae over the lower legs, became manifest. By comparing the respective data it will be seen that the ratio between the life span of man and that of the guinea-pig is roughly the same as the ratio between the corresponding times taken for the disappearance of the body ascorbic acid and for the onset of scurvy in these two species. For approximate comparison the factor of 10 may be adopted for converting the data obtained with guinea-pigs into those applicable to human subjects. When this factor is applied to the figures obtained in this investigation with guinea-pigs, taking into consideration at the same time that the vitamin C requirement of man is about double that of the guinea-pig [cf. Kellie & Zilva, 1939], it would appear that 120 mg. every 30 days would be sufficient to protect man from macroscopic scurvy, since 6 mg. every third day is required to produce this effect in the guinea-pig. A multiple of the daily dose of 10 mg., which corresponds to 5 mg. with guinea-pigs, would fulfil this function for man when given at even much longer intervals. Though no precision can be attached to these figures, they nevertheless indicate that doses of the order of 100 mg. given at intervals of a number of days or even weeks can afford at least minimum protection against scurvy. By utilizing the vitamin C requirements of the guinea-pig as a basis for calculation it was found that the dose for the human being could be deduced from them [Kellie & Zilva, 1939], the dose of about 15 mg. per day being found in this way to be necessary to protect man from scurvy. Apart from popular experience recent direct experiments on human beings suggest that this dose is of the right order [Rietschel & Mensching, 1939; Fox & Dangerfield, 1940; Widdowson & Alington, 1941]. There is good reason to believe that the indications obtained in this present investigation are also correct.

If these deductions are correct it may be considered what bearing they have on practical nutrition. As far as the civilian population of this country, leading a normal life, is concerned, the natural supply of vitamin C during the greater part of the year is so superabundant that, even allowing the widest margin for destruction in the cooking and the preparation of the food, the intake is more than adequate to supply the vitamin C requirements. Towards the end of the winter and the early spring, when green vegetables become rather scarce, some individuals, chiefly in urban areas, have to rely on potatoes as their chief source of vitamin C. In all probability this main source alone, considering the quantities that are usually eaten, together with the traces of ascorbic acid in other constituents of the diet, is sufficient to meet the vitamin C requirements of a great many people. When, in addition, it is considered that large quantities of vitamin C are often consumed intermittently during the winter and spring in the form of occasional greens, swedes, turnips, Bramley's Seedling apples, strawberry or black currant jam etc. and, in peace-time, citrus fruits, and when it is further realized that, as the above experiments indicate, a lasting effect can be produced by such irregular consumption of ascorbic acid, confidence is increased in the belief that a general vitamin C deficiency, even at that time of the year, need not

be greatly feared. It is quite probable that saturation may not be attained by many during winter and spring, but, as already pointed out by the author and his colleagues [Johnson & Zilva, 1934; Zilva, 1936; 1937; Kellie & Zilva, 1939], there is no evidence that saturation in respect to vitamin C is a prerequisite of adequate nutrition. The importance of saturation has had a great deal of ventilation in the literature during the last few years, but on close examination it is found that these claims are based on undigested and misinterpreted experiments. Much more convincing are some recent and carefully controlled investigations that militate against the exaggerated importance of saturation. Mensching [Rietschel & Mensching, 1939], after subsisting on a scorbutic diet for about 40 days, developed a cold as a result of exposure to a draught after becoming overheated by performing strenuous storm troop exercises. This infection cleared up normally. Crandon [Crandon *et al.* 1940] throughout an experimental period of $5\frac{1}{2}$ months, which included winter months, on a scorbutic diet enjoyed perfect freedom from respiratory infection, although in previous winters he was subject to such infection. Fox & Dangerfield [1940] also found no indication that a large group of unsaturated subjects was more prone to infection than another group which received more vitamin C. In all three investigations the gums of the subjects remained unaffected. In the last two investigations (in the first the point was not investigated) unsaturation did not influence the healing of wounds; only at the end of 182 days, when scurvy was manifest, did Crandon find that the healing of a self-inflicted wound was impaired. In Crandon's experiment it took 3 months of existence on a completely vitamin C-free diet before signs of fatigue became noticeable. Mensching did not observe any fatigue after living on a scorbutic diet for 100 days, when the experiment ended, nor did tests of physical efficiency in Fox & Dangerfield's investigation reveal any influence of unsaturation on the performance of the experimental subjects. Finally it may be mentioned that the author [Zilva, 1919] some years ago demonstrated that guinea-pigs on a scorbutic diet showed no differentiation in the amboceptor and agglutinin titres and in the complement content of the blood as compared with animals receiving ample vitamin C. All this evidence tends to show that the absence of full saturation, which may possibly occur after intermittent consumption of large doses of ascorbic acid capable of preventing scurvy, need not cause any serious anxiety.

I should like to take this opportunity of expressing my gratitude to Dr R. G. Hatton, Director of East Malling Research Station, for laboratory accommodation. Thanks are also due to Messrs Roche Products, Ltd., for a gift of ascorbic acid.

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139. THE BIOCHEMISTRY OF THE GAS GANGRENE TOXINS

1. ESTIMATION OF THE α TOXIN OF *CL. WELCHII*, TYPE A

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UNTIL recently the lethal factor, α toxin, occurring in the toxic filtrates of *Cl. welchii*, type A, cultures has been estimated mainly by two methods, namely, the determination of toxicity by intravenous injections into mice, and the determination of haemolytic activity *in vitro*. In addition the necrotic activity has been determined by intradermal injections into guinea-pigs. These three methods have been described by Ipsen *et al.* [1939]. As means of specifically estimating α toxin these methods are open to various objections. (For a distinction of the known toxins of *Cl. welchii*, type A, see R. G. Macfarlane *et al.* [1941], Dalling & Stephenson [1941] and the next paper in this series.) Trevan [1927] has given a detailed account of the errors unavoidably associated with the determination of toxicity by the mouse intravenous method. Apart from its inaccuracy this method also suffers from the fact that large numbers of mice are required, which are expensive and difficult to obtain. The estimation takes 2 or 3 days, and, moreover, is not specific in that it is a measure of the combined lethal activity of α toxin and such other lethal toxins as might occur with it.

The *in vitro* haemolytic assay of the α toxin is more rapid and less expensive than the mouse intravenous method, but has, until recently, been obscured by at least one other haemolysin, the θ toxin, in the toxic filtrates.

In work on the purification and separation of the toxins of *Cl. welchii* filtrates it is necessary to have an accurate, specific and convenient method for the determination of α toxin. The basis for such a method has recently been found by Nagler [1939] who observed a specific reaction between α toxin, but not toxoid, and normal human serum. This reaction is shown by the development of an opalescence soon after the reactants have been mixed, and he has used it in the determination of α toxin and of α antitoxin, and also of α toxoid [Nagler, 1941]. His method consists in determining the least amount of toxin (in toxin, toxin-antitoxin, or toxin-toxoid-antitoxin mixtures) which will give an observable degree of opalescence with the serum after 16 hr. incubation. For toxin determinations a series of dilutions of the unknown toxin is compared with a series of dilutions of a standard toxin, and, similarly, appropriate controls are used in the toxoid and antitoxin estimations. R. G. Macfarlane *et al.* [1941] have since shown that a similar reaction takes place between α toxin and the crude lecitho-vitellin solution obtained by filtering a suspension of egg yolk in isotonic saline. This solution is more sensitive to the toxin than normal human serum, and the reaction requires the presence of free calcium ions. These workers also estimate α toxin by observing the least amount of toxin which will bring about an opalescence, but not a flocculation, with the egg yolk solution after incubation overnight. The present writer has confirmed that toxoid formed by treating *Cl. welchii* toxin with formalin does not give this reaction.

In the method described below a departure is made from the procedure of observing the lowest amount of toxin which will bring about a noticeable reaction; instead, the degree of turbidity developed in a given time is correlated with the amount of toxin.

Materials and apparatus

The egg-yolk solution is made by beating the yolk of a day-old egg in 500 ml. of isotonic (0·9 %) saline which is 0·005 *M* with respect to calcium acetate. After about 20 g. kaolin have been stirred in, the suspension is filtered by gravity through ordinary filter paper, and the filtrate sterilized by passing through a Seitz E.K. or Ford G.S. filter pad. The sterile solution, which is slightly opalescent, and which varies in colour from blue-yellow to fairly deep yellow, is then stored in the refrigerator in 25 ml. amounts in sterile cotton wool-plugged test-tubes.

The standard toxin is prepared by dialysing fresh toxic filtrate against pure glycerol for 48 hr. at 0°, as described by M. G. Macfarlane & Knight [1941]. This glycerinated toxin is sterile and retains its activity undiminished for at least 9 months.

When turbidities were compared with an absolute standard a neutral grey filter of density 1·5, made by the Ilford Research Laboratory, was used in conjunction with a Leitz colorimeter. This colorimeter is fitted with a green filter in the eyepiece, and its plungers are coupled and therefore move into the blank and unknown solutions to the same depth. The neutral grey filter is placed in the path of the beam of light which has passed through a blank consisting of egg-yolk solution which has been incubated with isotonic saline. The other light beam passes through the unknown turbidity.

When unknown turbidities are compared with the turbidity produced by the standard toxin an ordinary Bausch and Lomb colorimeter is used. The eyepiece is fitted with the blue filter provided by the makers as well as with a Wratten B tricolour green filter. While readings are being taken extraneous light must be prevented from falling on the walls of the colorimeter cups by using the light guard provided with the colorimeter. Any good colorimeter is suitable, but it is recommended that one fitted with a built-in and adjustable light source be used.

The toxin-egg-yolk solution reaction

When amounts of toxin corresponding to 1–6 M.L.D. are incubated for 15 min. at 38° with 2 ml. of the egg-yolk solution fairly dense turbidities are produced, which fall outside the range of a nephelometer, but which can be conveniently measured in an ordinary colorimeter. Thus if 6 ml. of a toxin solution containing 1 M.L.D./ml. are incubated with 2 ml. egg-yolk solution for 15 min. at 38°, and the resultant turbid suspension is diluted, the degree of turbidity, as compared with the neutral grey filter, density 1·5, is directly proportional to the dilution. This is shown in Fig. 1. The abscissa shows the degree of dilution of turbidity and the ordinate the reciprocal ($1/d$) of the depth in mm. of turbidity corresponding to density 1·5.

The degree of turbidity produced by a given amount of toxin varies with different eggs, and with the age of the egg solution. In Fig. 2 the values of $1/d$ for density 1·5 are plotted against the amount of toxin used. Curves I and II were obtained with the same egg-yolk solution, but in II the egg-yolk solution was 24 hr. older than in I. In curve III a different egg-yolk solution was used. In all three curves the same toxin (ammonium sulphate-precipitated) was used.

In order to exclude variations due to different eggs and to the age of the egg-yolk solutions, the turbidities produced by different amounts of toxin were compared with the turbidity produced by an arbitrary unit of the standard toxin. An arbitrary egg unit (E.U.) was chosen as a fixed amount of the standard

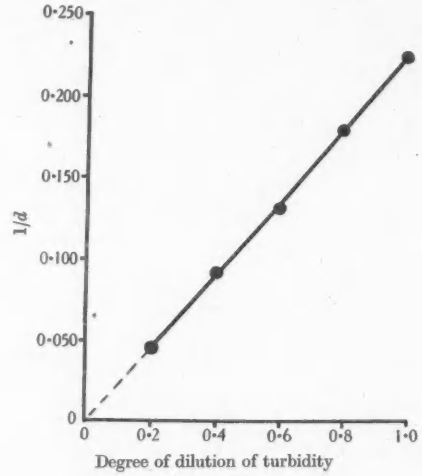

Fig. 1. Effect of dilution on turbidity.

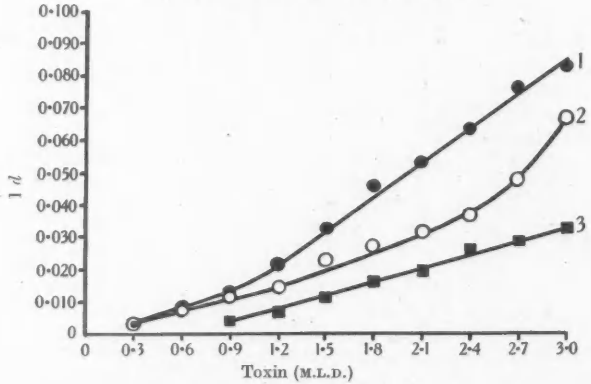

Fig. 2. Turbidity produced with different egg-yolk solutions compared with an absolute standard.

toxin corresponding to approximately 2 M.L.D. The activity curve shown in Fig. 3 was prepared as follows. The standard toxin was diluted with isotonic saline to give a solution containing 3 E.U./ml. Amounts of this dilution from 0·15 to 1·0 ml. were pipetted into a series of test tubes and in each case the toxin solution was made up to 1 ml. with isotonic saline. To each tube 2 ml. of the egg-yolk solution were then added, and the toxin-egg-yolk solution mixtures were incubated at 38°. After 15 min. the tubes were taken from the bath, and t

each of them 5 ml. of an isotonic saline solution containing 2–3 antitoxin units/ml. were added. The antitoxin solution was added to stop the reaction and to increase the turbid suspension to a convenient volume for use in the colorimeter. The turbidities obtained with the various amounts of toxin were then compared in the colorimeter with the turbidity produced by 1 E.U. of the standard toxin. In Fig. 3 the ratios of the reading for the standard turbidity to the readings for the unknown turbidities (S/U) are plotted against the amounts of toxin. It will be seen that over a threefold range (0·7–2·1 E.U.) the curve is linear and the degree of turbidity is directly proportional to the amount of toxin. During the summer months of this year (1941) all the eggs used gave the same activity curve, which did not change until the egg-yolk solutions were 7–10 days old,

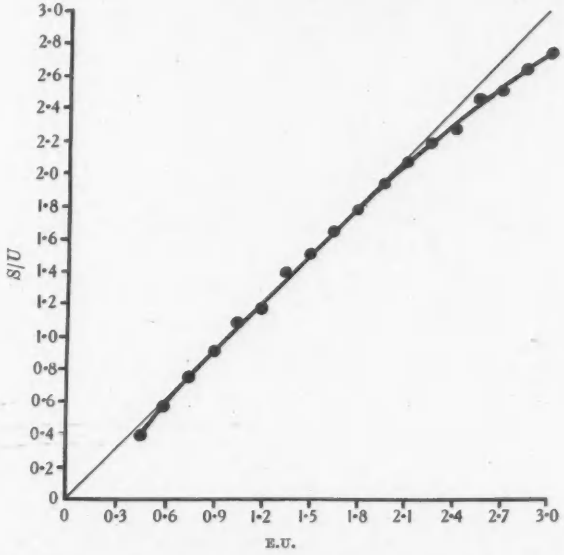

Fig. 3. Activity curve. Effect of toxin concentration on turbidity produced.

provided that the solutions were kept sterile and in the refrigerator. Lately (September, October) it has been noticed that, while all fresh egg-yolk solutions give the curve in Fig. 3, the shape of the curve varies rapidly with the age of the solution, sometimes after 4 or 5 days, often sooner. Occasionally an egg is still found which gives a constant curve for at least a week, and all egg-yolk solutions do not vary in the same way. It seems that while the blue-yellow egg-yolk solutions give less absolute turbidity, they are not more variable than the more yellow solutions. The variability in the recent eggs is either seasonal, or due to the war-time diet of the hens.

The 15 min. incubation period used in the construction of the activity curve was chosen because, as can be seen from Fig. 4, the reaction is still proceeding at constant velocity after this time. In Fig. 5 it will be seen that 2 ml. of the egg-yolk solution are sufficient to 'saturate' the toxin under the conditions employed.

If equal amounts of toxin and egg-yolk solution are incubated together in different dilutions, and the resultant turbid suspensions are then all diluted to

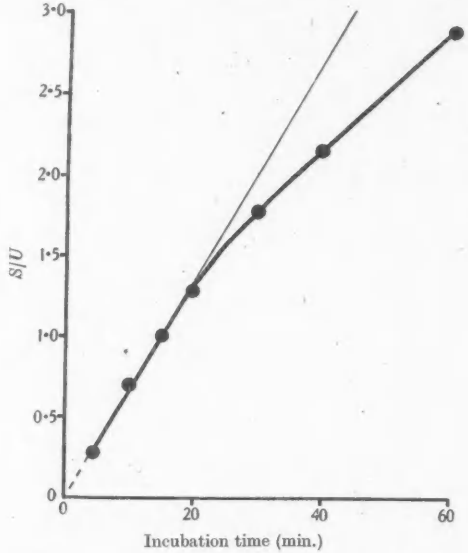

Fig. 4. Course of the reaction between 1 E.U. of toxin and 2 ml. of egg-yolk solution.

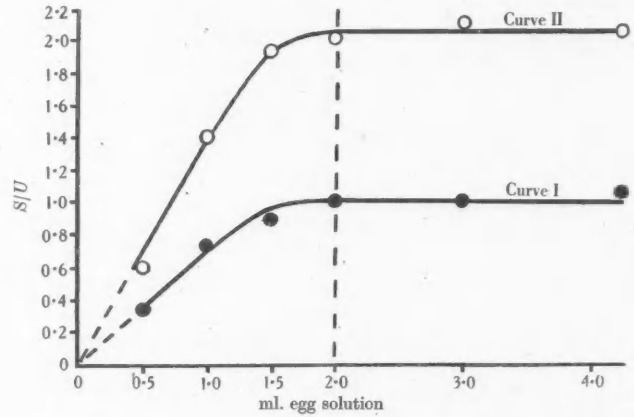

Fig. 5. Rate of the toxin-egg-yolk solution reaction as a function of 'substrate' concentration. Curve I with 1 E.U. of toxin. Curve II with 2 E.U. of toxin.

the same volume, it will be found that the turbidity produced decreases with the increasing dilution during incubation. This is shown in Fig. 6.

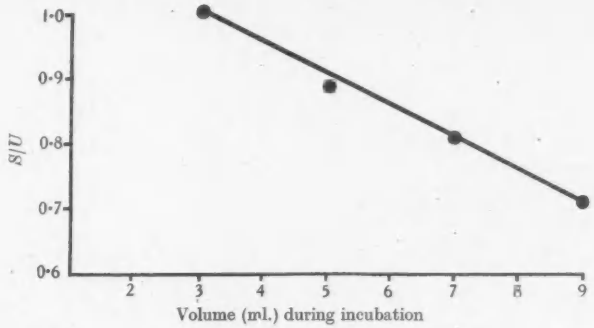

Fig. 6. Effect of dilution on development of turbidity by 1 E.U. of toxin with 2 ml. egg-yolk solution.

Estimation of α toxin

The method now employed for the estimation of α toxin is the following.

Three or more 1 ml. volumes of different dilutions of the toxin are incubated in test tubes with 2 ml. of the egg-yolk solution for 15 min. at 38°. Then 5 ml. of the isotonic saline solution containing 2–3 antitoxin units/ml. are added to each tube and the resultant turbid suspensions compared in the colorimeter with the turbidity produced by 1 ml. of a solution of the standard toxin containing 1 E.U. With a good colorimeter the readings can be easily matched with an error of not more than 2%. From the ratio S/U thus determined the corresponding number of E.U. for each amount of toxin can be read off from the activity curve. In this manner a triplicate determination of the activity of the unknown toxin in E.U./ml. can be obtained. If the activity of the toxin is entirely unknown it might, of course, be necessary to carry out a preliminary orientation with a series of twofold dilutions. If it is found that the three values for E.U./ml. do not agree, but decrease with decreasing amounts of toxin used, then the activity curve is no longer being obeyed. During the summer months the activity curve was obeyed for at least a week, and if a fresh egg-yolk solution was made up every week this difficulty was very rarely encountered.

In Table 1 some typical results obtained by this method are shown. The maximum difference in a set of triplicate readings is seldom $>8\%$, and generally $<5\%$. The last set of three values shown in this table is typical of those obtained with an egg-yolk solution which is no longer obeying the activity curve in Fig. 3. When such results were obtained during the summer months the egg-yolk solution was discarded, but with the recent eggs this has not been feasible, as the solutions often vary from day to day in the activity curve obtained. This difficulty can easily be surmounted by plotting a new activity curve on each day when it is desired to carry out estimations. The time required to obtain a daily 10-point activity curve does not exceed 45 min. Alternatively, when only a few estimations of activity are required, the activity curve need not be used, and the value of E.U./ml. can be determined by extrapolation. That is, 4 or 5 dilutions of the unknown toxin, some containing less and some more than 1 E.U. are incubated with the egg-yolk solution and the turbidities compared with the standard turbidity. The results are then plotted, and by extrapolation the amount of toxin which gives the same turbidity as 1 E.U. can be inferred.

Table 1. *Triplicate values obtained in determinations of α toxin*

Average E.U./ml.	Toxin used ml.	*S/U*	E.U.	E.U./ml.	Max. difference %
36·5	0·0500	1·79	1·82	36·4	3·3
	0·0375	1·35	1·35	36·0	
	0·0250	0·93	0·93	37·2	
38·3	0·0500	1·86	1·90	38·0	4·2
	0·0375	1·41	1·41	37·6	
	0·0250	0·98	0·98	39·2	
171	0·0067	1·20	1·20	179	8·2
	0·0050	0·84	0·84	168	
	0·0033	0·50	0·55	165	
192	0·0067	1·32	1·32	193	1·5
	0·0050	0·95	0·95	190	
	0·0033	0·64	0·64	192	
203	0·00500	1·00	1·00	200	3·0
	0·00375	0·78	0·78	206	
	0·00250	0·45	0·55	204	
235	0·0100	2·28	2·40	240	4·3
	0·0075	1·70	1·72	230	
	0·0050	1·18	1·18	236	
239	0·0067	1·57	1·57	234	4·2
	0·0050	1·22	1·22	244	
	0·0033	0·80	0·80	240	
1013	0·0033	3·00	3·40	1020	6·0
	0·0025	2·44	2·60	1040	
	0·0017	1·64	1·64	980	
—	0·0500	2·72	3·00	60	—
	0·0375	2·03	2·10	56	
	0·0250	1·19	1·19	48	

Determination of combining power (test dose)

In preliminary work on the determination of the combining power of toxins by means of the turbidimetric method described above it was found that when low concentrations of toxin and antitoxin were used the results were anomalous. Fig. 7 shows the results obtained when a series of 4 ml. volumes, each containing 3 E.U. toxin/ml. and 0·04–0·08 antitoxin unit/ml., were left to stand together at room temperature for 1 hr. After this period 1 ml. aliquots from each 4 ml. volume were incubated with 2 ml. egg-yolk solution, and the residual activity was determined by the method described above. The values thus obtained were subtracted from 3 to give the number of E.U. of toxin neutralized by the various amounts of antitoxin. The three curves shown are the results of three apparently identical experiments carried out on the same day. While the amount of toxin neutralized by a given amount of antitoxin varies considerably, it will be seen that in each case a given increase in the amount of antitoxin is equivalent to the same increase in the amount of toxin neutralized. Thus the increase of 0·04 antitoxin units, from 0·04 to 0·08, corresponds to an increase of 1·07, 1·13 and 1·12 E.U. of toxin neutralized in curves A, B and C, respectively, the average being 1·11 E.U. From the value 1·11 E.U./0·04 antitoxin unit it can be calculated that 0·2 unit of antitoxin neutralize 5·55 E.U. of toxin. The L+/5 value, or test dose, of a toxin, as determined by the mouse intravenous method, is defined as that amount of toxin which, in the presence of 0·2 unit of antitoxin will kill some, but not all, of the mice injected. Since 1 E.U. is approximately 2 M.L.D., the test dose of the toxin can be calculated to be approximately

6·05 E.U. (5·55 E.U. + 1 M.L.D., or 0·5 E.U.) or, since the toxin used contained 200 E.U./ml., 0·030 ml. The L+/5 value for the toxin, as determined by the mouse intravenous method, was 0·024 ml.

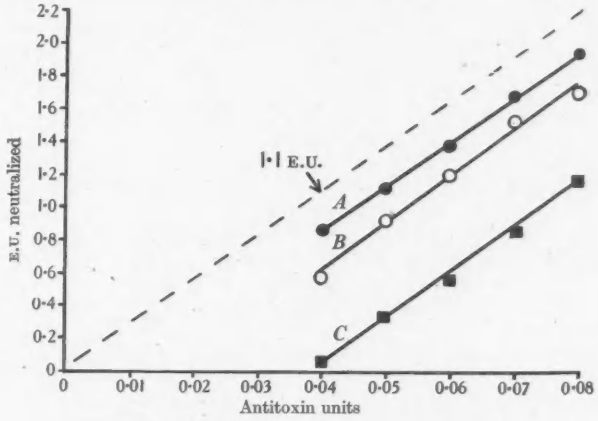

Fig. 7. Neutralization of toxin by antitoxin in low dilution.

It was found that when the concentrations of toxin and antitoxin in the mixtures were increased the results obtained were more reproducible, and, under the conditions employed, the toxin-antitoxin combination seemed to take place in simple stoichiometric proportions. Triplicate values for test dose of a toxin could be carried out in a little over an hour by setting up a series of tubes, each containing in a 1 ml. volume 0·2 antitoxin unit and increasing amounts of the toxin. After 30 min. at room temperature 2 ml. of the egg-yolk solution were added to each of the toxin-antitoxin mixtures and the residual activity of the toxin was determined in the usual way. A typical experiment is shown in Table 2.

Table 2. *Determination of combining power*

ml. toxin dilution containing 10 E.U./ml.	0·4	0·5	0·6	0·7
ml. antitoxin dilution containing 1 unit/ml.	0·2	0·2	0·2	0·2
ml. isotonic saline	0·4	0·3	0·2	0·1
E.U. toxin added	4·0	5·0	6·0	7·0
E.U. found remaining	0	0·6	1·5	2·6
E.U. neutralized by 0·2 antitoxin unit	—	4·4	4·5	4·4
		Average 4·43		

Test toxin contains 44 E.U./ml.
Test dose 4·43 E.U. or 0·10 ml.

The test dose is here defined, not as that amount of toxin which, in the presence of 0·2 antitoxin unit, contains a limited amount of free toxin, but as that amount of toxin which is just completely neutralized by 0·2 antitoxin unit. The antitoxin used in these experiments was the Standard Gas Gangrene (Perfringens) Antitoxin issued by the Department of Biological Standards of the National Institute of Medical Research at Hampstead.

Determinations of combining power carried out by the method outlined above have so far proved to be entirely satisfactory, and no anomalous results have been obtained. It must be emphasized, however, that so far only about 10 determinations have been carried out, and this number is not large enough to establish the efficacy of the method.

Moreover, in the toxins so far studied, the toxin-toxoid ratio must either have been high, or the affinities of the toxin and toxoid for antitoxin must have been the same; otherwise the relationship of toxin neutralized to amount of antitoxin used could not have been a simple stoichiometric one. In such cases the combining power cannot be calculated directly by subtracting the values for residual E.U. from E.U. added; but if the values for residual E.U. are plotted against the E.U. added it will presumably be possible to determine by extrapolation the amount of toxin just neutralized by 0·2 antitoxin units.

The antitoxin concentration in these experiments was 0·2 unit/ml. and in the experiments illustrated in Fig. 7 it varied from 0·04 to 0·08 unit/ml.; it is possible that the anomalous results of the latter experiments were due to dissociation of the toxin-antitoxin complex at the lower concentrations.

Estimation of antitoxin

Conditions have not yet been found for reliable and reproducible estimation of antitoxin, but the results so far obtained are worthy of discussion. The fairly typical results shown in Fig. 8 were obtained with a test toxin containing 938 E.U./ml. and having a test dose of 3·75 E.U., and with an α-antitoxin, kindly provided by Mr Glenny of the Wellcome Physiological Research Laboratory, containing 100 anti-α-units/ml. as estimated in his laboratory by an *in vitro* haemolytic method.

A series of 1 ml. volumes, each containing 1 test dose of toxin and increasing amounts of antitoxin, were left at room temperature for 30 min.; 2 ml. of the egg-yolk solution were then added to each tube and the residual activities of the toxin-antitoxin mixtures determined in the usual way. From these figures the percentage test dose neutralized by each amount of antitoxin was calculated. The results obtained from three apparently identical experiments are plotted in Fig. 8. If the value of 88% test dose neutralized by 0·001667 ml. antitoxin, which is common to all three curves, is taken, the value of the antitoxin can be calculated as follows:

$$88\%\text{ test dose} = 0{\cdot}001667\text{ ml. antitoxin},$$

$$1\text{ test dose} = 0{\cdot}001667 \times \frac{100}{88} = 0{\cdot}0019\text{ ml. antitoxin},$$

$$= 0{\cdot}2\text{ antitoxin units},$$

i.e. antitoxin contains 105 units/ml.

By a similar calculation the other two figures common to curves II and III (36 and 38% test dose neutralized by 0·000667 ml. antitoxin) give a value of 109 units/ml. The broken straight line in Fig. 8 represents a simple stoichiometric reaction between antitoxin (105 units/ml.), and toxin (test dose 3·75 E.U.) in which the toxin-toxoid ratio is high, or the affinities of toxin and toxoid for antitoxin equal. The value 105–109 antitoxin units/ml. is in excellent agreement with the value of 100 units/ml. given by Mr Glenny, especially in view of the fact that these values were found by different workers using entirely different methods with different test toxins.

It is difficult to see why the results obtained in the estimation of antitoxin should be so anomalous when the results of the estimations of combining power

were regular and consistent. It is, of course, possible that the regular results of the combining power estimations were fortuitous. Another possible explanation might lie in the fact that anomalous results have, in the author's experience, always been found when the concentration of antitoxin was appreciably below 0·2 unit/ml. This is shown in Figs. 7 and 8. In Fig. 8 it will be seen that the values of toxin neutralized are inconsistent when the antitoxin concentration falls below 0·001667 ml. antitoxin/ml., or approximately 0·175 unit/ml. In the

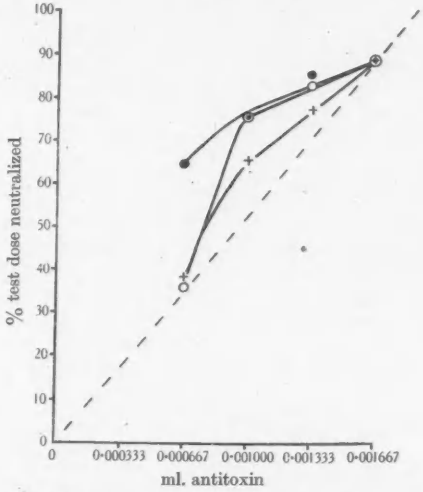

Fig. 8. Neutralization of 1 test dose by increasing amounts of antitoxin.

estimations of combining power the antitoxin concentration was kept at 0·2 units/ml., and in the estimation of test dose and antitoxin by the mouse intravenous method, as recommended by the Department of Biological Standards, the antitoxin concentration is of the order of 0·4 unit/ml. It is interesting to note that in Fig. 7, comparing curves B and C with curve A, the tendency is for a given amount of antitoxin to neutralize less toxin, whereas in Fig. 8, the antitoxin appears to show a tendency to neutralize more toxin than the amount represented by the 'ideal' curve.

Discussion

The work so far done on the estimation of combining power and of antitoxin must be regarded as preliminary, and is being extended. It is possible that when concentrations of antitoxin of the same order as those used in the mouse intravenous method are employed more consistent results will be obtained.

The method described above for the estimation of toxin has proved to be of great convenience and satisfactory accuracy in the author's experience extending over about 9 months. It has however, been applied to toxins from one strain only, namely, S 107, and its efficacy can only be established when it has been successfully used by a number of different workers using toxins produced by different strains. It has the advantage over the methods described by Nagler [1939] and by R. G. Macfarlane *et al.* [1941] that the activity of several samples can be determined within an hour, with very satisfactory accuracy, and without

resorting to a large number of serial dilutions. It is particularly useful in studying the toxin-antitoxin reaction since only one tube need be used in the determination of the residual activity in a given toxin-antitoxin mixture. Apart from the saving of time, the short period of incubation employed also enjoys the advantages pointed out by Haldane [1930] associated with the measurement of reaction velocity while the velocity is still constant. The fundamental principles of the methods described in this paper and in that of Macfarlane *et al.* are the same, since in both cases the amount of toxin which produces a given degree of turbidity is determined. In the one case this degree of turbidity is defined in terms of a standard toxin, and in the other it is that amount produced under given conditions which is observable but which does not flocculate: therefore difficulties associated with turbidity determination, such as changes in particle shape or size, are common to both methods. In the author's experience such difficulties have apparently not been encountered.

SUMMARY

1. A turbidimetric method for the estimation of α toxin is described which is based on the reaction between egg-yolk solution and toxic filtrates of *Cl. welchii*, type A.

2. The application of this method to the estimation of combining power and of antitoxin is discussed.

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140. THE BIOCHEMISTRY OF THE GAS GANGRENE TOXINS

2. PARTIAL PURIFICATION OF THE TOXINS OF *CL. WELCHII*, TYPE A. SEPARATION OF α AND θ TOXINS

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THE existence of a number of toxins in the filtrates of the classical gas gangrene organism, *Cl. welchii*, type A (*perfringens*) has been recognized in recent years. Henry [1922], working with *Cl. welchii* isolated from man, distinguished between a 'haemotoxin' and a lethal 'myotoxin'. Wilsdon [1931; 1932–3] classified *Cl. welchii* into four distinct types, and Glenny *et al.* [1933] considered the toxin of type A to be a single entity. They established the parallelism of lethal, haemolytic and necrotic activities of the toxin, which they called α, to distinguish it from the β, δ and ϵ toxins of types B, C, and D. Prigge [1937] claimed to have separated by fractional precipitation with ammonium and sodium sulphates two fractions, one 'ζ' which was lethal and poorly haemolytic, and another 'α' which was haemolytic and hardly lethal. The existence of six toxins has been claimed by Weinberg *et al.* [1937], but serologically these toxins can be grouped into three antigenically distinct toxins, each showing one or more active properties [Ipsen & Davoli, 1939]. One of these three antigens, the η toxin, does not occur regularly, and so far has been found in appreciable quantity only in the filtrates of the French Lechien strain grown in a special medium; abundant antibody is, however, apparently present in Danish and British antisera [Ipsen, 1939; Weinberg & Guillaumie, 1938]. The other two, now named α and θ, apparently occur quite regularly in the toxic filtrates of *Cl. welchii*, type A, but in variable proportions. The properties of these toxins have been summarized by Ipsen & Davoli [1939] as follows:

Toxins*	Haemolysis of sheep cells	Lethality to mice	Dermal reaction
α (ζ)	+	+ +	Necrosis
θ (α)	+ + +	(+)	(Haemorrhagia)
η (η)	0	+	0

* The letters in brackets are those used by Ipsen & Davoli; it has recently been agreed by a group of English workers that this nomenclature is unfortunate, and the non-bracketed names are now used [Dalling & Stephenson, 1941].

In addition to its haemolytic, lethal and dermo-necrotic properties the α toxin is also very probably responsible for the specific reaction which takes place when toxin and normal human serum or egg-yolk solution are mixed. Its capacity to haemolyse and to react with egg-yolk solution is dependent upon the presence of free calcium ions [R. G. Macfarlane *et al.* 1941]. M. G. Macfarlane & Knight [1941] have produced evidence that indicates that an enzyme, lecithinase, which they found in toxic filtrates of *Cl. welchii*, type A, is identical with the α toxin.

The existence of an unstable haemolysin has been known since 1925. Neill [1926], working with unclassified (probably type A) *Cl. welchii* filtrates, found a haemolysin which was reversibly inactivated when the toxin was left exposed to air. The haemolytic activity could then be restored by the addition of various reducing agents. Since his haemolytic experiments were carried out in phosphate buffer the haemolytic activity of the calcium-activated α toxin was completely suppressed (see below); Neill was therefore probably estimating the activity of the θ toxin, which was first mentioned as such in print by R. G. Macfarlane *et al.* [1941]. Todd [1941] has since demonstrated the close relationship between θ toxin and the oxygen-sensitive haemolysins of streptococci, pneumococci and *Cl. tetani*. θ toxin is neutralized by high titred antistreptolysin O sera, and the antibody to θ toxin neutralizes streptolysin O. Although these oxygen-sensitive haemolysins are closely related serologically, they are not antigenically identical.

Little work has been done on the purification and separation of the toxins of *Cl. welchii*. Prigge [1937] claimed to have separated a 'ζ' toxin, with a high ratio of lethal to haemolytic activity, from an 'α' toxin with a high ratio of haemolytic to lethal activity. The separation was effected by fractional precipitation with ammonium and sodium sulphates. R. G. Macfarlane *et al.* [1941] have already pointed out that the haemolytic activity of Prigge's 'ζ' toxin was probably suppressed owing to his use of a phosphate buffer. It is also important to note that Prigge did not treat his 'ζ' and 'α' toxins with reducing agents before measuring their haemolytic activities. If a reducing substance, originally present in his crude filtrates, appeared predominantly in his 'α' fraction, then his 'ζ' fraction might still have contained large proportions of his 'α' fraction, but in a reversibly inactivated form. Ipsen *et al.* [1939] point out that the divergences in comparative titrations of various *perfringens* antitoxins with Prigge's 'ζ' and 'α' toxins do not amount to more than 30%.

In this paper the partial purification of the toxins of *Cl. welchii* type A is reported, as well as the separation of the α and θ toxins.

Methods and materials

(1) *Culture medium.* The medium described by M. G. Macfarlane & Knight [1941] was used, with the exception that the peptone solutions were sterilized by passing through a 14 cm. Ford G.S. pad, rather than by steaming on three successive days.

(2) *Preparation of toxic filtrates. Cl. welchii*, type A, strain S 107, was used throughout. For purposes of inoculation into the culture medium the strain is maintained in cooked meat tubes, and the inoculations are made from fresh 16 hr. cultures. About every 3 months the activity of the culture filtrates starts to decrease; then the current cultures in the meat tubes are discarded, and new cultures are obtained by inoculating meat tubes from quiescent sealed cultures on alkaline egg medium [Robertson, 1916]. These egg tubes are inoculated from the active cultures of known toxicity and, after suitable anaerobic incubation (4 days), can be preserved in sealed tubes indefinitely. If this is done the activity of the culture filtrates can be kept at a constant level of about 90 M.L.D./ml. Cultures are grown in 2 l. volumes in round-bottomed flasks, without special anaerobic precautions. The cotton wool plugs are tied down to prevent their being pushed out by the vigorous gassing. After 6 hr. incubation in a water bath at 38° the *p*H of the cultures is about 6·0. To filter the cultures they are adjusted to *p*H 7·0 with *N* NaOH and about 20 g. kaolin per litre are stirred in; the resultant suspensions filter rapidly under gravity through pleated filter paper to give crystal clear filtrates.

(3) *Estimation of activity.* Lethal activity is determined by intravenous injection of 0·5 ml. volumes of various dilutions into the tails of 17–20 g. mice. For each determination of median lethal dose five dilutions are used, spaced about 20 % apart, and each dilution is injected into five mice. Generally the practice is to determine the number of E.U./ml. of the toxin and then to inject doses corresponding to 156, 125, 100, 80 and 64 % of 0·5 E.U. into the 5 groups of 5 mice (1 E.U. is generally equivalent to 2 M.L.D. [van Heyningen, 1941]). The percentages of deaths in each group after 48 hr. are then plotted against the corresponding doses and the median lethal dose taken as that amount of toxin which corresponds to 50 % deaths.

α Toxin is also determined by the turbidimetric method described in the preceding paper.

Haemolytic activity is determined by a method similar in principle to that described by Herbert [1941]. Five or six 1 ml. volumes of dilutions spaced about 20 % apart are incubated at 38° in 15 ml. centrifuge tubes with 1 ml. volumes of an approximately 6 % suspension of sheep red blood cells. After 30 min. incubation 5 ml. of an isotonic saline solution, containing 2–3 antitoxin units/ml., are added to each of the tubes to stop further action by the haemolysin. The unhaemolysed cells are centrifuged, and the supernatant fluids, containing varying amounts of liberated haemoglobin, are carefully poured off into test tubes. The degree of haemolysis is then compared in a colorimeter with a standard which is equivalent to 50 % haemolysis. The comparisons are made in a Bausch and Lomb colorimeter which is fitted with the blue filter provided by the makers, and with a Wratten B tricolour green filter. The percentage haemolysis is plotted against the amounts of haemolysin used, and by extrapolation the amount of haemolysin which will cause 50 % haemolysis is inferred. An arbitrary haemolytic unit (H.U.) is defined as that amount of haemolysin which will cause 50 % haemolysis in the approx. 6 % suspension of sheep red blood cells after 30 min. incubation at 38°. A typical activity curve for haemolysis is shown in Fig. 1.

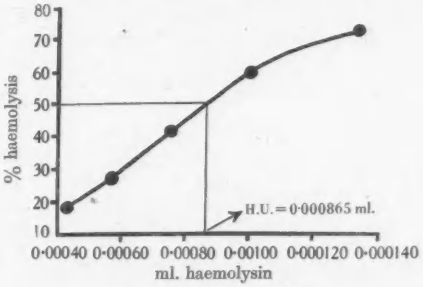

Fig. 1. Typical haemolysis curve.

The standard approx. 6 % suspension of sheep red blood cells is made as follows. The sheep's blood is defibrinated by shaking with glass beads and the red cells are centrifuged and washed twice by resuspension in volumes of isotonic (0·9 %) saline equal to double the original volume of blood. A suspension of the cells is then prepared by making up a 25 ml. volume of the packed cells to 100 ml. with isotonic saline. 0·5 ml. of this approx. 25 % suspension is laked by adding 9·5 ml. of distilled water. The resultant haemoglobin solution is then compared with a neutral grey filter, density 1·5, in a Leitz colorimeter fitted

with a green filter in the eyepiece [see Herbert, 1941]. The solution should be of such a strength that a depth of 5·8 mm. is equivalent to density 1·5; if it is too strong the original suspension is diluted accordingly with isotonic saline. The value of 5·8 corresponds to a haemoglobin solution of 0·4 %, as determined in a spectrophotometer; the approx. 25 % suspension of red blood cells is therefore standardized to contain 8 % haemoglobin. This suspension is kept in a refrigerator and diluted fourfold with isotonic saline just before use. A suspension is never kept for more than 5 days.

The 50 % haemolysis standard is prepared by laking 0·5 ml. of the approx. 6 % suspension with 6·5 ml. of distilled water.

When haemolysis by θ toxin is determined the dilutions of toxin are made in isotonic phosphate buffer *p*H 6·5. The phosphate completely suppresses the haemolysis by α toxin, which requires the presence of free calcium ions. Reversibly inactivated θ toxin is reactivated by reduction with $M/25$ thiolacetic acid; 2 ml. of the toxin are left at room temperature for 15 min. with 0·5 ml. of $M/5$ neutralized thiolacetic acid, and the dilutions in the phosphate buffer are made with this mixture.

As will be seen below, it was found that haemolysis by α toxin is of the 'hot-cold' type. In the estimation of the haemolysis by α toxin dilutions are made in isotonic saline, *p*H 6·5, and 0·005 M with respect to calcium acetate, so that the concentration of calcium in the toxin-red blood cell mixtures is 0·0025 M. After the 30 min. incubation period ice-cold isotonic antitoxin solution is added to the tubes, which are then placed in ice water for 30 min. before being centrifuged. Otherwise the procedure is the same as that described above for θ toxin. Very little liberation of haemoglobin takes place after the 30 min. period at 0°. Estimations by this method also include the activity of active θ toxin.

(4) *Carbon estimations* are carried out in the Van Slyke manometer by the wet combustion method of Van Slyke & Folch [1940]. Generally 0·5 or 1 ml. aliquots of the toxin solutions are used; the water is evaporated off by gently boiling the solutions in the combustion tube with a few pieces of quartz, and with continual agitation over a small flame. The error is not greater than 1 %. Estimations can be carried out directly on solutions which are 1/5 saturated with ammonium sulphate. In such cases an error of about +5 % is observed.

Partial purification of the toxins

The conventional media usually consist of hot-water extracts of meat, containing peptone and cooked meat, and the toxins are generally precipitated from them by 2/3 saturation with ammonium sulphate. The activity of the filtrates from these media is of the order of 3 M.L.D./mg. dry weight, which probably includes quite considerable amounts of ammonium sulphate. For the purification of the toxin of *Cl. welchii* it was necessary, therefore, to find a medium different from those which have hitherto been employed. The medium described by M. G. Macfarlane & Knight [1941], besides yielding highly active filtrates with great regularity, contains comparatively little non-dialysable or ammonium sulphate-precipitable material. The activity of the filtrates is of the order of 30 M.L.D./mg. dry weight of non-dialysable material. Thus a purification of about 10-fold can be obtained by using this medium. By the method described below the toxin is purified from M. G. Macfarlane & Knight's medium with 50 % yield and by a factor of nearly 40-fold, or, very roughly, 400-fold with respect to the non-dialysable material in the usual culture media. Precipitation of the toxins from the filtrates is very unsatisfactory since mechanical losses in the collection of the precipitate (about 1 g./l.) are comparatively large; moreover,

the amount of toxin soluble in the ammonium sulphate solution is probably appreciable in comparison with the total amount of material precipitated. Therefore it seemed desirable to concentrate and purify the toxins by absorption from the crude filtrates. This can be done as follows.

Method I.

1000 ml. toxic filtrate.
75 ml. *M*/5 phosphate buffer *p*H 6·5.
650 ml. acetone.
15 ml. *M* calcium acetate.

The filtrate is cooled to 0° in a jar immersed in ice and salt, *M*/5 phosphate buffer *p*H 6·5 is added and then acetone at −10° is slowly poured in, with constant stirring, until its concentration is 40 %. The temperature must not rise above 2–3°, as the toxin is otherwise destroyed. *M* calcium acetate is then slowly added with stirring. The resultant precipitate of calcium phosphate adsorbs the toxin. After standing at 0° for at least 15 min. the precipitate is centrifuged and eluted twice with successive 50 ml. volumes of 1/5 saturated ammonium sulphate solution in water, and the two eluates are combined. The eluate is light yellow and contains 50–60 % of the toxin, purified about 10-fold.

This procedure is recommended only for small quantities of toxin since it is difficult and tedious to maintain a low enough temperature when working with large volumes.

Unless acetone is used the toxins are not adsorbed on the calcium phosphate, but if the filtrate is first dialysed the concentration of acetone can be lowered to 20 %; this concentration does not injure the toxins and consequently the adsorption can be carried out at room temperature.

For large volumes of filtrate the following procedure is therefore used.

Method II. The filtrate is dialysed overnight (16 hr.) in wide cellophane tubing ('Visking' sausage casing, 40 mm. wide when flat) against running tap water. Adsorption is then carried out with the following proportions of materials:

1000 ml. dialysed filtrate.
75 ml. *M*/5 phosphate buffer *p*H 6·5.
250 ml. acetone.
15 ml. *M* calcium acetate.

The acetone is added slowly at room temperature, with constant stirring. After the calcium acetate has been added the adsorption is allowed to proceed for 15 min. and the calcium phosphate is then collected. When large volumes are used it is most convenient to use a Sharples centrifuge which can be operated continuously. The rotating cylinder of the centrifuge must have holes in the top so arranged that only the 'supernatant' fluid is ejected; the precipitate is spun on to the wall of the cylinder, which is lined with hardened filter paper. The liquid enters the rotating cylinder from the bottom through a 3·5 mm. delivery jet, under a head of about 75 cm., and under these conditions passes through the centrifuge at the rate of about 1 l. per min. The calcium phosphate precipitate is small in relation to the total volume of fluid and settles in a thin layer on the filter paper. The rapid flow through the 3·5 mm. delivery jet ensures that the precipitate is distributed over the total height of the filter paper liner, and does not clog the lower part of the cylinder. The 'supernatant' still contains a little precipitate. After the fluid has passed through the centrifuge once via the 3·5 mm. delivery jet, it is sent through again, without stopping the centrifuge, via a 1·5 mm. jet. In this way the precipitate is quantitatively collected at an

overall rate of flow of about 250 ml./min. It is easy to deal with 10 l. or more in this manner. After centrifuging, the filter paper liner is carefully withdrawn from the cylinder and spread out on a sheet of glass. The precipitate is then scraped off with a table knife and suspended in a volume of 1/5 saturated ammonium sulphate solution equal to 1/20 the original volume of filtrate. The precipitate is finely suspended and spun down in an ordinary centrifuge, and the elution repeated. The two eluates are combined to give a light yellow solution containing 70–80 % of the toxin, purified over 10-fold.

The toxin in these eluates can be further purified and concentrated by a second adsorption and elution. The eluate is dialysed for 6 hr. against running tap water, and then, since the volume is conveniently small, subjected to the procedure in method I. It is more convenient at this stage to concentrate 5-fold rather than 10-fold; a further 3-fold purification is effected and the yield is again 70–80 %. It is important not to wash the calcium phosphate precipitates with water, since this would remove quite a large proportion of the toxins.

Typical results of a double adsorption and elution are shown in Table 1.

Table 1. *Purification of toxin by adsorption and elution*

Preparation	Volume ml.	E.U./ml.	mg. C/ml.	E.U./mg. C	Overall yield %	Overall purification	Test dose E.U.
Crude filtrate	2500	45	17	2·6	—	—	4·43
Dialysed filtrate	3000	37·6	1·09	34·4	100	—	—
1st eluate	250	324	0·76	427	72	12·4-fold	—
2nd eluate	50	1215	0·91	1336	54	38·8-fold	3·95

The degree of purification is with reference to the dialysed filtrate.

The decrease of the toxin in the test dose probably indicates that a small degree of toxoid formation has taken place during the purification.

The solution containing 1215 E.U./ml. is light yellow, and, in 1/5 saturated ammonium sulphate solution, retains its activity for at least 10 days at 0°. After it has been dialysed the toxin solution loses its activity rapidly. The most convenient way to keep the toxin is to dialyse the eluate overnight, and then to dry it from the frozen state. This procedure does not cause any loss in activity, and results in a light white product which is easily soluble in water or isotonic saline. The carbon content of the final product is about 50 %, and the M.L.D. is about 0·75 μg.

The second eluate contains about 2 mg. of non-dialysable material per ml., and does not give a precipitate with trichloroacetic acid, or when saturated with solid ammonium sulphate. On dialysis against a saturated solution of ammonium sulphate, however, a precipitate is obtained. The possibilities of further purification along these lines are being investigated.

Separation of α and θ toxins

It was found that under suitable conditions θ toxin can be quantitatively adsorbed from a mixture of α and θ toxins. If 2 ml. undiluted sheep red blood cells are mixed with 8 ml. of fresh toxic filtrate and left for 5 min. at 0°, the supernatant, after the red cells have been centrifuged, will show no θ activity, as measured in isotonic phosphate buffer, even after reduction with thiolacetic acid. If the centrifuged red cells are resuspended in isotonic saline at 38° they are lysed almost immediately. The α toxin content of the supernatant, as measured by the turbidimetric method, is unchanged, or slightly diminished,

but the haemolytic activity when measured in calcium saline is high. If the filtrate is left with the red cells for longer than 5 min. more α toxin will be adsorbed, but the adsorption of the α toxin apparently does not begin until all the θ toxin has been adsorbed. The adsorption of θ toxin can also be carried out with smaller amounts of red cells, but then the time of adsorption must be increased in proportion. Fig. 2 shows the effect of time on the adsorption of θ

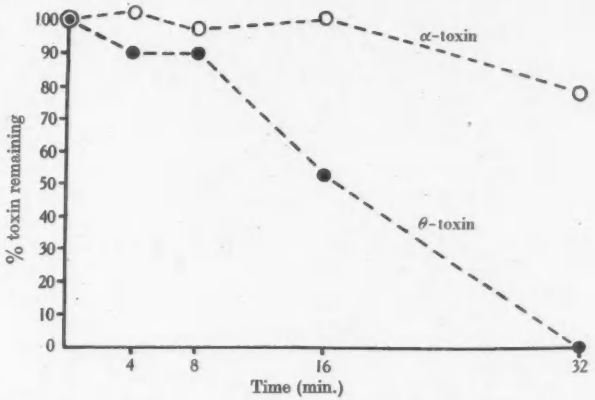

Fig. 2. Effect of time on adsorption of α and θ toxins by red blood cells.

and α toxins when 2 ml. amounts of fresh filtrate containing 48·5 E.U./ml. were left with 0·1 ml. amounts of undiluted washed sheep red blood cells at 0°; α toxin was determined by the turbidimetric method, and θ toxin was determined in phosphate buffer after reduction with thiolacetic acid. During adsorption a very slight degree of haemolysis may take place, in which case a small amount of liberated haemoglobin will appear in the supernatant. θ toxin can also be adsorbed from diluted (60 E.U./ml.) solutions of the partially purified toxins. The θ toxin content of the purified fractions has not yet been determined quantitatively, nor has the adsorption of θ toxin from high concentrations of toxins been studied. The haemolytic activity of the θ-free preparations is completely inhibited by low concentrations of Glenny's α antitoxin, and unaffected by high concentrations of his θ antitoxin.

The conditions outlined above for the adsorption of θ toxin hold only for fresh filtrates. When 8 ml. of a filtrate which had stood for 10 days at 0° were treated with 2 ml. of undiluted washed sheep red blood cells for 5 min. at 0°, only about 70 % of the θ toxin was adsorbed. The θ activity of the fresh filtrate was 471 H.U./ml., and after reduction with thiolacetic acid it was the same, viz. 480 H.U./ml. The θ activity of the 10-day-old filtrate was 23 H.U./ml., but on reduction it was restored to 473 H.U./ml. Reduction of the stale filtrate and consequent reactivation of the θ toxin did not bring about a greater adsorption of θ toxin. Nor was more than 70 % adsorbed when the proportion of filtrate to red blood cells was changed from 8 : 2 to 5 : 5, or when the adsorption time was increased from 5 min. to 30 min.

This incomplete adsorption of θ toxin from stale filtrates, as compared with the quantitative adsorption from fresh filtrates, is inexplicable. It is apparently

not due to conversion of θ toxin into toxoid, since its activity could be completely restored by reduction; nor can it be due to the reversible inactivation of θ toxin since the reactivated toxin was not adsorbed to a greater extent than the inactivated toxin. An extended investigation of the adsorption is obviously desirable.

It was found that α toxin is a 'hot-cold' haemolysin. This interesting type of haemolysis has been known for some time in connexion with the staphylococcus β haemolysin [see Glenny & Stevens, 1935; Roy, 1937, and Smith & Price, 1938]. Ipsen *et al.* [1939], working with a toxin-antitoxin mixture, found that no haemolysis took place after 2 hr. at 37°, but that haemolysis did take place during a subsequent 18 hr. period at room temperature. The toxin was one of Prigge's, with a low ratio of haemolysin to lethal toxin, and presumably contained a high ratio of α toxin, or θ toxin in reversibly inactivated state. These workers state that the effect observed could not be compared with the 'hot-cold' lysis of staphylococcus β haemolysin since it was not observed with the toxin alone. They ascribe the effect to a probable dissociation of the toxin-antitoxin complex at low temperature.

When a suitable concentration of the α and θ toxins in calcium saline was incubated for 30 min. at 38° with a 6% suspension of sheep red blood cells there was no apparent haemolysis; if 5 ml. of isotonic antitoxin solution at 38° were added there was still no haemolysis, but if 5 ml. of antitoxin solution at room temperature, or at 0°, were added, complete haemolysis took place very rapidly. Complete haemolysis also took place when the incubated mixture of toxin and red blood cells was cooled in ice. α toxin which has been freed of θ toxin by adsorption exhibits the same phenomenon, but if the concentration of α is sufficiently high complete haemolysis will take place during the incubation period. θ toxin is an ordinary 'hot' haemolysin; haemolysis by solutions of toxic filtrate in phosphate buffer, in which α is inactive, is not of the 'hot-cold' variety.

The lysis which takes place during the cold period is apparently independent of further action by the α toxin. Two equal amounts of α toxin were incubated with the red blood cell suspension for 30 min. at 38°; to one mixture were added 5 ml. of an isotonic solution containing 3 antitoxin units/ml., and to the other 5 ml. of ordinary isotonic saline. After 1 hr. at 0° the degree of haemolysis was estimated and found to be the same in both tubes.

In the estimation of α toxin it is therefore necessary to cool the toxin-red blood cell mixtures before the degree of haemolysis is determined. The 30 min. period at 0°, mentioned in the section on methods and materials, was chosen because very little further haemolysis takes place after this time.

Table 2 shows typical values obtained in the determinations of haemolysis by the α and θ toxins of *Cl. welchii*, type A, strain S 107. The preparation in sections 5 and 9, the θ-free filtrate, was obtained by treating 8 ml. of fresh filtrate with 2 ml. undiluted washed sheep red blood cells for 5 min. at 0°. After the red cells had been centrifuged the supernatant had no θ activity and the α toxin content, as determined by the turbidimetric method, had decreased from 47·7 to 43·5 E.U./ml. All the values in the table refer to the same filtrate.

It will be seen that the haemolytic activity of a mixture of α and θ toxins (α+active θ, or α+reactivated θ) is no greater than that of α alone (or α+inactivated θ). When two series of dilutions, both containing the same concentration of α toxin, but one containing θ toxin as well, are incubated with red cells, the series with the θ toxin will show haemolysis in some of the dilutions and the series without θ toxin will not; but if both series are cooled the extent of haemolysis will be found to be the same in both. At first it was thought that this

Table 2

	Preparation	Toxins involved	H.U./ml.
1	Fresh filtrate, in phosphate buffer	Active θ	471
2	Fresh filtrate after reduction, in phosphate buffer	Active θ	480
3	10-day-old filtrate in phosphate buffer	Inactivated θ	23
4	10-day-old filtrate, after reduction, in phosphate buffer	Reactivated θ	473
5	Fresh filtrate, after adsorption with R.B.C., in phosphate buffer, with or without reduction	None	0
6	Fresh filtrate, in calcium saline, by 'hot-cold' method	α + active θ	1145
7	10-day-old filtrate, in calcium saline, by 'hot-cold' method	α + inactivated θ	1100
8	10-day-old filtrate, after reduction, in calcium saline, by 'hot-cold' method	α + reactivated θ	1000
9	θ-free filtrate, in calcium saline, by 'hot-cold' method, corrected for partial loss of α	α alone	990

phenomenon was due to the inhibition of α by θ, by competition for the red blood cells. The fact that θ toxin is adsorbed by the red cells before α toxin seemed to support this view. It was thought that every α unit inhibited by θ was counterbalanced by a θ unit, i.e. 1000 α units + 500 θ units would amount to 500 active α units + 500 inhibited α units + 500 active θ units = 1000 active haemolytic units. If this were the case, then reversibly inactivated θ toxin should not be adsorbed by the red blood cells; otherwise the values of the preparation 7, α + inactivated θ, should be lower than that of α alone, or of α + active θ. In fact, as was mentioned above, inactivated θ is adsorbed to the extent of 70%. The fact that the combined haemolytic activities of α and θ toxins are not additive therefore remains to be explained.

With the exception of a filtrate which is discussed below, the values in Table 2 are typical of the filtrates so far examined. The ratio H.U./E.U. in untreated filtrates, and in filtrates from which the θ has been adsorbed, has always been from 20 to 23, and the ratio of M.L.D./E.U. in the same preparations, has, within the limits of the errors of the assay for toxicity, always been approximately 2.

Recently a filtrate, apparently prepared in the same manner as the previous one, was obtained which gave the results shown in Table 3. The filtrate contained 43·5 E.U./ml. and its activity was completely inhibited by *Cl. welchii*, type A antitoxin. It differs from the previous filtrates in the following respects:

(1) The θ activity is apparently doubled (preps. 1–4).

(2) After adsorption with red blood cells there is still some activity (prep. 5). This activity does not require the presence of free calcium ions, and is unstable since it disappears after 4 days (prep. 6); nor can it be recovered by reduction with thiolacetic acid (prep. 7). This apparently unstable activity is again shown in the difference between 1686 H.U./ml. for prep. 8, and 1430 H.U./ml. for preps. 9 and 10.

(3) In preps. 9, 10 and 11 the ratio H.U./E.U. is about 31, as compared with the ratio about 21 previously found.

These results can be explained by assuming that two more haemolysins have appeared. For convenience in discussion these hypothetical haemolysins will be called X and Y.

Table 3. *Properties of filtrate possibly containing additional toxins*

	Preparation	Hypothetical toxins involved	H.U./ml.
1	Fresh filtrate, in phosphate buffer	Active θ	958
2	Fresh filtrate, after reduction, in phosphate buffer	Active θ	1018
3	4-day-old filtrate, in phosphate buffer	Partially inactivated θ	455
4	4-day-old filtrate, after reduction, in phosphate buffer	Reactivated θ	1000
5	Fresh filtrate, after adsorption with R.B.C., in phosphate buffer	X	200–300
6	Same as 5, after 4 days, in phosphate buffer	Inactivated X	0
7	Same as 6, after reduction, in phosphate buffer	Inactivated X	0
8	Fresh filtrate, in calcium saline, by 'hot-cold' method	Active $\theta + X + \alpha + Y$	1686
9	4-day-old filtrate, in calcium saline, by 'hot-cold' method	Partially inactivated $\theta + \alpha + Y$	1430
10	Same as 9, after reduction, in calcium saline, by 'hot-cold' method	Reactivated $\theta + \alpha + Y$	1430
11	Preparation 6, in calcium saline, by 'hot-cold' method, corrected for partial loss of α	$\alpha + Y$	1360
12	α, calculated from H.U./E.U. = 21 and E.U./ml. = 43·5	α	915

X is an unstable haemolysin, which is not adsorbed by red blood cells; it is unlike α in that it does not require the presence of free calcium ions, and its loss of activity, unlike that of θ, cannot be restored by reduction. It contributes 200–300 H.U./ml.

Y is a more stable haemolysin, which is also not adsorbed by red blood cells; it is apparently associated with α, and seems to require the presence of free calcium ions. In the 4-day-old preparations it increases the H.U./E.U. ratio from 20 to about 30. It contributes about 500 H.U./ml. (the difference between the values in sections 11 and 12).

If these assumptions are correct, then the haemolytic activities of $\alpha + X + Y$, unlike those of $\alpha + \theta$, are additive.

The lethal activity of the filtrate was not determined. These findings with the latest filtrate must be treated with reserve, as they have been observed with only one filtrate. They are mentioned here in case other workers should not be able to confirm the results set out in Table 2. The discussion below is not concerned with this latest filtrate.

Discussion

It is interesting to examine some of the earlier work on the toxins of *Cl. welchii* in the light of the results reported above. Numerous workers in the past [e.g. Glenny *et al.* 1933] have demonstrated a close parallelism of the lethal and haemolytic properties of the toxic filtrates. As it has since been demonstrated that there are at least two haemolysins, one of which (θ) is only slightly lethal, and easily inactivated, it might be considered surprising that it has been possible to demonstrate such parallelism. The results here reported, however, show that, provided that the haemolytic activity is estimated by a 'hot-cold' method, and not in phosphate buffer, the value obtained is always that of the α toxin. (This might not hold if there were more θ units/ml. than α units.) It appears to be

independent of the amount of θ toxin, and of its state of activation. Glenny *et al.* did their haemolytic estimations in isotonic borate buffer, and they took their readings after 2 hr. incubation and overnight standing at room temperature. Standing overnight at room temperature is probably sufficient to complete 'hot-cold' haemolysis.

Other workers have adsorbed the toxins on red blood cells. Thus Wuth [1923] treated 3 ml. of *Cl. welchii* filtrate with 3 ml. undiluted sheep's red blood cells for 2 hr. at 0°, and found that all the haemolytic activity had been removed. His method of estimating haemolysis was such that it probably included haemolysis by α toxin. Since he used a very high concentration of red cells, and since he left the toxin-red cell mixture to stand for 2 hr. it is probable that he adsorbed both θ and α toxins. He was also able to adsorb all the haemolysin on powdered fibrin. When he treated an unstated amount of toxin with 0·02 ml. of 1% methyl alcohol solution of lecithin, 90% of the haemolytic activity was lost. This was conceivably due to competitive inhibition of the α toxin by lecithin, which M. G. Macfarlane & Knight [1941] have shown to be a substrate for an enzyme which is probably α toxin.

Henry [1923] found that when fresh toxic filtrates of *Cl. welchii* were treated with thick suspensions of red blood cells for 24 hr. at 0°, there was a decrease in lethal activity. When stale filtrates were treated similarly there was no decrease in lethal activity. It was shown above that red cells will adsorb all of the θ, and some of the lethal α toxins from fresh filtrates, but only about 70% of the θ, and none of the α, from stale filtrates. Henry also showed that a 'myotoxin', presumably the α toxin, could be adsorbed on fresh chopped guinea-pig muscle.

It is obvious that there is still a great deal to be done in connexion with the toxins of *Cl. welchii*, and the work reported in this paper can only be regarded as preliminary. In view of the close relationship of streptolysin O and θ toxin, it would be interesting to determine whether the combined haemolytic activities of streptolysin O and α toxin are non-additive. Smith & Price [1938] found that the addition of broth or glycerol to dilutions of staphylococcus β haemolysin abolished the 'hot-cold' haemolytic effect; with glycerol the same end point was obtained at 37° as in 'hot-cold' lysis, and with broth the end point was slightly displaced. It is necessary to see whether the same effect can be observed with the α toxin of *Cl. welchii*.

While it is possible to obtain θ-free preparations of α toxin by adsorption with red blood cells, it would be convenient to find another adsorbent, preferably inorganic. Red blood cells are not very suitable for the adsorption of θ toxin on a large scale. Moreover, it is not possible to recover the θ toxin from the red blood cells. A few inorganic adsorbents, namely kaolin, kieselguhr, alumina C_γ and dialysed iron have already been tried, but without success.

Formal proof that the factors which are responsible for the 'hot-cold' haemolysis, the lethal action, the necrotic action, the reaction with egg-yolk solution and the lecithinase activity are identical is probably impossible to provide. There is, however, much evidence that these varied activities are, in fact, due to one toxin, the α toxin. M. G. Macfarlane & Knight [1941] have demonstrated the parallelism of toxicity, development of turbidity with egg-yolk solution and lecithinase activity. They found this parallelism not only in various samples, but also in samples which had been partially inactivated by bubbling with nitrogen. The present writer has similarly demonstrated parallelism of toxicity, turbidity production and 'hot-cold' haemolysis. Parallelism has also been demonstrated for necrotic and lethal activities (see, for example, Glenny *et al.* [1933]). The demonstration of parallelism of necrotic and lethal activities

with other activities cannot be very close because of the inaccuracies associated with their assay.

The objection to the type of evidence just discussed is that two or more separate toxins having similar physical properties and showing different activities could also exhibit such parallelism. But if several different antisera were assayed against the same toxin preparation by measurement of the above-mentioned five types of activity, and if these five methods all gave concordant values for each of the antisera, then that would be convincing evidence that the five types of activity are due to a single toxin. If these activities were due to different toxins it would be extremely unlikely that their respective antitoxins would occur in corresponding proportions in an antiserum, and even more unlikely that these proportions would hold for a number of antisera. Moreover, the percentage error in the mouse intravenous method, or in the necrotic assay, is much lower in antitoxin or test dose determinations than in straight determinations of activity, so that lethal or necrotic activity could be correlated more exactly with other activities. Parallelism of the various properties by antitoxin assay has already been established by a number of workers [see, for example, Glenny *et al.* 1933; Nagler, 1939; 1941; R. G. Macfarlane *et al.*, 1941; and M. G. Macfarlane & Knight, 1941]. It is desirable, however, that work on these lines with θ-free toxin be extended.

Finally it must be emphasized that all the work reported in this paper was done with a single strain; obviously it is necessary that other strains should be studied. Crook [1941] has demonstrated parallelism of toxicity and reactivity with egg-yolk solution and normal human serum in 40 batches of filtrates from 14 type A strains.

Summary

1. The toxins of *Cl. welchii*, type A, have been partially purified.
2. θ toxin has been separated from α toxin.
3. α toxin has been shown to be a 'hot-cold' haemolysin.
4. The combined haemolytic activities of α and θ toxins are not additive.

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141. THE ELIMINATION OF 3:4-BENZPYRENE FROM THE ANIMAL BODY AFTER SUBCUTANEOUS INJECTION

2. CHANGED BENZPYRENE

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After the subcutaneous injection of 3:4-benzpyrene in the rat, two fluorescent elimination products have been detected in the faeces and urine: unchanged benzpyrene and a derivative of benzpyrene. In previous communications, the fluorescent derivative of benzpyrene extracted from bile was called 'BPX' and that from faeces 'BPF'. The fluorescence spectra of unpurified extracts of 'BPX' and 'BPF' showed two bands in the blue-violet region of the spectrum, but there was a slight shift in the position of the bands. However, after purification by solution in NaOH and adsorption on alumina, concentrates of 'BPX' and 'BPF' showed the same fluorescence spectrum, and it appeared that the substances were identical. The expression 'BPX' is therefore used in this communication to denote the fluorescent derivative of benzpyrene extracted from the faeces as well as from the bile.

In a quantitative experiment [Chalmers & Kirby, 1940] it was found that approximately 1 % of the injected benzpyrene was eliminated unchanged in the faeces of the rat, while only a trace was eliminated in the urine. The method of fluorescence spectrum analysis used in the estimation of benzpyrene was not so suitable for the estimation of 'BPX', which shows two wide bands in the blue-violet region of the spectrum, in contrast to benzpyrene, which shows a number of sharp bands. Consequently, there is no quantitative evidence of the relative amounts of 'BPX' eliminated in the urine and faeces, but the qualitative evidence indicates that only a small fraction of the injected benzpyrene is eliminated as 'BPX', and that 'BPX' is eliminated in relatively larger amounts in the faeces than in the urine. A more detailed study of the properties of 'BPX' has been made, and the results of this work are presented here.

Experimental

Rats were injected subcutaneously with a 1 % solution of the hydrocarbon in olive oil. The animals were kept in metabolism cages, and the faeces and urine collected separately. Most of the animals developed tumours at the site of injection and were thereupon killed. Nephritis and lung abscesses found in some of the injected animals, and at first thought to be due to the injections, were also found to a similar extent in control animals of the same stock. The livers of injected animals examined *post mortem*, appeared normal, although subsequent histological examination revealed toxic changes.

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The method of extraction of the fluorescent material from the faeces was as described by Chalmers [1940] and Chalmers & Kirby [1940]. The 'BPX' was separated from unchanged benzpyrene by solution in dilute NaOH, and by chromatographic adsorption on alumina. A concentrate of 'BPX' was sublimed at 150–160° at 0·01 mm. pressure, when a small quantity of yellow needle-shaped crystals was obtained, together with a fluid distillate. The fluid distillate, which was probably a decomposition product of 'BPX', could not be separated from the crystalline sublimate under the conditions used: a better yield of crystalline material would possibly result from the use of a higher vacuum.

Melting point of 'BPX'. Only a small quantity of 'BPX' was available for examination, and it was therefore necessary to use a micro-M.P. apparatus. The crystals on a microscope cover glass were placed on a metal disk floating on a bath of Wood's metal, and the M.P. observed through a microscope. The apparatus was tested with a number of substances of known M.P., and the values found were correct $\pm 1°$. A sample of 4′-hydroxy-3:4-benzpyrene (prepared by Prof. Cook) thus examined, softened at 214° and melted sharply at 217°, in agreement with the recorded value. With substances such as 'BPX' and 6-hydroxy-3:4-benzpyrene, which sinter and decompose on melting, in order to reduce the effects of sintering on the M.P. determination the bath of Wood's metal was allowed to reach a temperature of about 20° less than the expected value before the compound was placed on the apparatus.

The M.P. of the crystals of 'BPX', 190–196° decomp., corresponded with that of 6-hydroxybenzpyrene recorded by Fieser & Johnson [1940]. A sample of their compound, however, examined in our apparatus gave a lower value, probably due to the instability of the substance. A further investigation of the M.P. of 'BPX' and 6-hydroxybenzpyrene is recorded in the section on the crystallographic examination, by one of us (D. C.), and it is shown that these substances behave differently.

Fluorescence spectrum. The fluorescence spectra of 'BPX' and the hydroxybenzpyrenes in ethanol solution are as follows:

	Fluorescence spectrum bands Å.	
'BPX'	(4250–4400) (4500–4650)	Two bands moderately well defined
6-Hydroxy-BP	(4200–4350) (4400–4650)	Two bands less well defined than 'BPX'
4′-Hydroxy-BP	(4150–4700)	One wide band

In fluorescence spectrum, therefore, as well as in M.P., 'BPX' is not identical with 6-hydroxy- or 4′-hydroxy-benzpyrene.

Solubility of 'BPX' and hydroxybenzpyrenes.

Solvent	'BPX'	6-Hydroxybenzpyrene	4′-Hydroxybenzpyrene
2*N* NaOH	s-cold (green fluor.-soln.)	s-cold (red fluor. soln.)	s-cold (red fluor.-soln.)
N Na_2CO_3	s-cold	i-cold, s-hot	i-cold, s-hot
N $NaHCO_3$	i-cold, s-hot	i-hot	i-hot

For comparison the solubilities of 'BPX' and the known hydroxybenzpyrenes are tabulated above. The solubility of the substances was indicated by the fluorescence of the solutions in the ultraviolet beam. It will be seen that 'BPX' is slightly more acidic than the other compounds, but this difference may be due to the presence of the fluid distillate, associated with the crystals examined. It is therefore likely that 'BPX' is phenolic.

Crystallographic examination of 'BPX', 6-hydroxybenzpyrene and 4'-hydroxybenzpyrene

In an attempt to obtain additional evidence on the structure of 'BPX', samples of the sublimed substance were examined crystallographically. The examination was first undertaken to check the identity or otherwise of the preparation with 6-hydroxybenzpyrene and comparative observations were later made also on 4'-hydroxybenzpyrene.

All the preparations were available as very small samples of crystals. These were studied first under the microscope and subsequently X-ray measurements were made on 'BPX' alone.

Morphological and optical examination. The observations made on the optic and morphological character of the different crystals are tabulated below and further summarized by the diagram (Fig. 1). Optic figures were observed only in the case of preparations 1 and 3.

	Morphology	Optics
1. 'BPX'	Yellow laths on (001), elongated along [010]. Marked cleavage ‖ [010]	Extinction straight, $\alpha \parallel [010]$, β probably $\perp$ (001)
2. 6-Hydroxybenzpyrene:		
(*a*) From toluene	Fine yellow needles	Extinction oblique, 'slow' direction 47° to needle axis
(*b*) Sublimed	Very small laths	Extinction straight, 'slow' direction ‖ needle axis
3. 4'-Hydroxybenzpyrene	Yellow laths	Extinction straight, $\beta \parallel$ needle axis

It is clear from these data that the four preparations described are crystallographically different. Presumably 2(*a*) and 2(*b*) are polymorphic modifications of 6-hydroxybenzpyrene 2, of which 2(*a*) might contain solvent of crystallization. Since 1 and 2(*b*) were prepared in the same way, by sublimation, it

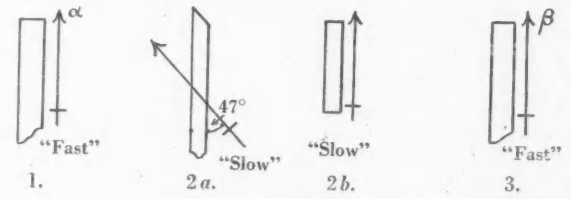

Fig. 1. Diagram to illustrate the optic and morphological character of crystals of (1) 'BPX'; (2*a*) 6-hydroxy-3:4-benzpyrene from toluene; (2*b*) 6-hydroxy-3:4-benzpyrene, sublimed; (3) 4'-hydroxy-3:4-benzpyrene. The extinction directions are shown by the arrows.

seemed unlikely that the 'BPX' crystals were also a polymorphic modification of 6-hydroxybenzpyrene, but to check this point mixed melting experiments were carried out under the microscope.

The melting and recrystallization were observed by using a hot wire stage [Bernal & Crowfoot, 1933]. A few crystals of 'BPX' were first melted on a cover slip by sliding this gradually towards a hot wire. On withdrawing the wire a short distance 'BPX' recrystallized in thin leafy plates, optically and morphologically identical with the original crystals. The process could be repeated with very little apparent decomposition of the preparation provided that the melting was carried out quickly and was not prolonged. The sample was then

melted and seeded with a crystal of 6-hydroxybenzpyrene, 2(*a*) (from toluene). 'BPX' crystals reformed on cooling and there was no growth of crystals of type 2(*a*).

Samples of both preparations of 6-hydroxybenzpyrene were then melted similarly. The melts rapidly discoloured and no recrystallization occurred on cooling either alone or after seeding with crystals of 'BPX'. It was clear also that the crystals melted at a lower temperature than 'BPX'.

X-ray crystallographic examination of 'BPX'. The fact that so far only a small quantity of very small crystals of 'BPX' has been available for study has limited the amount of information obtainable by the usual routine X-ray methods. It is not possible at present to attempt anything in the way of an X-ray analysis of the molecular structure, but an approximate measure of the mol. weight and some indication of the sort of molecular structure present can be gained even from the very limited observations possible.

The actual crystals used were approximately $1/10 \times 1/50 \times 1/250$ mm. in linear dimensions and were inclined to be bent. X-ray photography was therefore difficult, but photographs were obtained of three crystals with exposure times of 24 hr. for 15° oscillations. The photographs show few X-ray reflexions, and those tending to be blurred and drawn out owing to imperfections in the crystals. In the circumstances it is possible that the true unit cell is some multiple of that deduced from the present observations and that the space group indicated now is only a pseudo space group. The crystal symmetry may, in fact, not be orthorhombic but only monoclinic. But since even a determination of the pseudo cell and space group should provide useful information the measurements are recorded as follows:

Unit cell dimensions	$a = 8{\cdot}22$ Å.
	$b = 4{\cdot}34$.
	$c = 35{\cdot}58$ (or $c \sin \beta$).
Space group	*Pcb*.
Density	Between 1·375 and 1·402, nearer 1·402.

An attempt was made to measure the density of the crystals by flotation under centrifugal force in a solution of potassium mercury iodide but it proved too difficult to distinguish the very few very small crystals in the bulk of the solution. Individual crystals were therefore observed in drops of solution of known density under the microscope and their behaviour confirmed by their subsequent transfer into the solution in bulk. The limits between which the density must lie could certainly be refined by a more careful experiment, but since these limits appear close enough to fix the mol. weight the experiment was concluded.

Molecular weight. Assuming that there are four molecules in the unit cell as required by the space group found, and using the cell dimensions given above and a value for the density of 1·39, $M = 267$. This agrees well with the value 268 calculated for a monohydroxybenzpyrene.

Even allowing a probable error of 1 % on the X-ray measurements and taking the outside limits found for the density, the mol. weight may be calculated as lying between 278 and 258. These figures would exclude the possibility that the compound is a dihydroxybenzpyrene.

Molecular structure. The crystallography of the compound is in good agreement with the molecule having an aromatic structure of roughly the dimensions calculated for a derivative of benzpyrene. The *b* unit cell dimension is short,

4·34 Å., and since b is optically the direction of α, the least refractive index, it is reasonable to suppose that the molecules lie with the plane of the aromatic ring system approximately in the b plane. The area defined by the lengths of a and c is roughly that required by four times the area of the benzpyrene rings suitably packed together.

There is no evidence from the crystallographic data that 'BPX' is a hydroxybenzpyrene derivative beyond the fact that the mol. weight found is of the right order of magnitude. But if there is a hydroxyl group present, the arrangement of the molecules in the crystal must almost certainly be one in which the hydroxyl groups of neighbouring molecules are brought sufficiently close together for hydrogen bonds to be formed between them [cf. for example Robertson, 1940]. This consideration, combined with the admittedly rather doubtful space group data given above, restricts the probable position of the hydroxyl group among the number available within the benzpyrene molecule. The most likely position for the hydroxyl group would appear to be 7, 8, 9, 1′, 2′, or 3′, with 5, 6, 10, and 4′ just possible (of which 6 and 4′ are excluded by the direct comparison) and 1 and 2 very unlikely.

Discussion

The evidence that 'BPX' is a hydroxy derivative of benzpyrene is based on its solubility in dilute alkali. Only a small quantity of the crystalline material was available and even this was not obtained free from fluid distillate, which, as explained above, may have influenced the solubility and M.P. of the 'BPX'. It is hoped that a fuller examination of its properties may be made when more crystalline 'BPX' has been obtained.

A crystallographic examination of the 'BPX' at present available has shown that the mol. weight of the compound is of the right order of magnitude for a monohydroxy derivative of benzpyrene, assuming 'BPX' to be phenolic. In addition, consideration of the arrangement of the molecules in the crystal has indicated the most likely positions for the hydroxyl group in the molecule.

Spectrographic evidence has been obtained that benzpyrene is metabolized, at least in part, to a fluorescent derivative, by all the animals tested in this laboratory [Peacock, 1936; Chalmers & Peacock, 1936 and unpublished, and Chalmers, 1938]. In the mouse, rabbit, fowl and goat the carcinogen was introduced intravenously or subcutaneously; in the case of the rat, the hydrocarbon was also fed in the diet. The change of benzpyrene to 'BPX' was common to these different animals, and although the 'BPX' eliminated represented only a fraction of the injected benzpyrene, it is possible that 'BPX' is an intermediate compound formed in the metabolism of benzpyrene by the animal body.

The fluorescent elimination products of benzpyrene—unchanged benzpyrene and 'BPX'—do not appear to represent more than a fraction of the injected benzpyrene. Moreover, no evidence has been obtained of the elimination of benzpyrene or a derivative of benzpyrene in significant quantities conjugated with mercapturic acid or glucuronic acid. It therefore appears that most of the injected benzpyrene is eliminated as a non-fluorescent derivative or is further metabolized.

With regard to the elimination of benzpyrene in conjugation with glucuronic acid, it was found that after a subcutaneous injection of benzpyrene in the rat the urine gave a more intense glucuronic acid reaction with naphthoresorcinol than normal rat urine. Attempts to isolate the glucuronic fraction from the urine, however, were unsuccessful, and by comparison with the urine of rats fed with borneol it appeared that the glucuronic acid was present in small quantity.

SUMMARY

3:4-Benzpyrene, after subcutaneous injection in the rat, is metabolized to a fluorescent derivative which is probably a monohydroxybenzpyrene.

We are indebted to Prof. J. W. Cook and to Prof. Fieser for samples of 4′-hydroxy- and 6-hydroxy-3:4-benzpyrene.

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142. THE EXCRETION OF DERIVATIVES OF CERTAIN CARCINOGENIC AND NON-CARCINOGENIC HYDROCARBONS IN FOWL BILE

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THE fate of 3:4-benzpyrene after injection (subcutaneously and intravenously) in experimental animals has been described in previous publications from this Laboratory. A fluorescent derivative 'BPX' is excreted by the liver and may be recovered either from fistula bile or from the faeces [Peacock, 1936; Chalmers, 1938]. Subsequent fluorescence spectrum analysis has shown that about 1% of a quantity of benzpyrene injected subcutaneously in the rat was eliminated unchanged in the faeces, while only a trace was eliminated in the urine [Chalmers & Kirby, 1940]. Apart from this 1% of unchanged benzpyrene, the proportion excreted as 'BPX', or other derivatives, is not yet determined. Attempts to recover the fluorescent derivative 'BPX' have resulted in the isolation of minute amounts of a crystalline substance, whose properties suggest that it may be a monohydroxybenzpyrene [Chalmers & Crowfoot, 1941]. Earlier experiments with 1:2:5:6-dibenzanthracene had given no evidence of its excretion in the bile, either unchanged or as a fluorescent derivative, but the intravenous injection of methylcholanthrene and of anthracene gave rise to fluorescent substances in the bile [Chalmers & Peacock, 1936]. As will be shown in this communication, a derivative of dibenzanthracene with a faint blue fluorescence is excreted in the bile, but its detection by direct fluoroscopy is difficult.

In view of the finding that benzpyrene was partly excreted unchanged, investigation of a number of other hydrocarbons, both carcinogenic and non-carcinogenic (see Table 1), was undertaken by the method of sampling bile from fowls with enclosed cholecystostomy fistulae [Peacock, 1940].

EXPERIMENTAL

Methods. Colloidal 0·03–0·05% solutions of each hydrocarbon were prepared, and 5–6 ml. were injected intravenously into fowls with previously prepared biliary fistulae. It was found that the majority of the hydrocarbons tested gave rise to fluorescence of the bile within a few hours of the intravenous injection. For comparative purposes, solutions of the hydrocarbons in fowl serum and bile were tested, as described below, and in Table 1 the results of these tests are compared with the appearances of the fistula bile.

Bile was collected until the samples no longer showed any fluorescence in the ultra-violet beam (usually 1–2 days), in which time about 20 ml. of bile were obtained. The bile was extracted by a method similar to that used in the extraction of benzpyrene from the urine and faeces of rats [Chalmers, 1940; Chalmers & Kirby, 1940]. The bile was acidified with dilute HCl and extracted

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with ether, and the ether extract was then washed with dilute NaOH and water. Subsequent reference will be made to the examination of the alkali-soluble fraction. The ether extract was dried over Na_2SO_4, and the solvent evaporated in N_2 under reduced pressure. A benzene solution of the residue was filtered

Table 1. *Comparison of fluorescence of hydrocarbon solutions and fistula bile*

Hydrocarbon	Fluorescence of fowl bile + hydrocarbon, *in vitro*	Fluorescence of fowl bile after injection
Carcinogenic:		
1. 3:4-Benzphenanthrene	Blue-violet	—
2. 3:4-Benzpyrene	Violet	Blue-violet
3. 1:2:5:6-Dibenzanthracene	—	Faint blue
4. Cholanthrene	Violet	Blue
5. Methylcholanthrene	Violet	Blue
Non-carcinogenic:		
6. Anthracene	—	Faint green
7. Phenanthrene	—	—
8. Pyrene	Green-blue	Blue
9. 2′:6-Dimethyl-1:2-benzanthracene	—	Blue
10. 2′:7-Dimethyl-1:2-benzanthracene	—	Blue
11. Fluoranthene	Green-blue	Blue

through a 3–5 cm. column of alumina, to remove the bile pigments, giving a colourless filtrate: a small column was sufficient to remove the colouring matter. The alumina was washed first with benzene to remove any extraneous fluorescent material. The chromatogram filtrate which should contain any hydrocarbons present was evaporated to dryness and the residue dissolved in a small quantity of ethanol or benzene, for spectrographic analysis.

After intravenous injection in the fowl, benzpyrene was the only hydrocarbon examined which appeared unchanged in the bile. In view of the fact that benzpyrene is intensely fluorescent, it was possible that fluorescence spectrum analysis could detect smaller quantities of this compound in bile extracts than of other less intensely fluorescent compounds. To meet this possibility, extracts were made of normal bile to which 1 % or less of the quantity of 1:2:5:6-dibenzanthracene, methylcholanthrene and anthracene used for intravenous injection had been added, and it was found that in each case the fluorescence spectrum of the hydrocarbon in question could be detected. It may therefore be concluded that less than 1 % of the quantity of these hydrocarbons injected intravenously was eliminated unchanged in fowl bile.

After the intravenous injection of quantities of 0·03 % benzpyrene colloid varying from 1 to 10 ml., unchanged benzpyrene could be detected in the bile. In an attempt to find if there was a threshold below which benzpyrene would not be eliminated unchanged, smaller quantities of the hydrocarbon were injected, but the method of extraction and analysis was not considered satisfactory for experiments in which quantities of less than 0·3 mg. benzpyrene were injected.

Quantitative controls were not made with the other compounds examined, but it was found that they could be recovered unchanged from solution in fowl bile *in vitro* by the same method of extraction. It may be concluded, therefore, that after intravenous injection they were not eliminated unchanged in the fowl, or that they were eliminated unchanged in relatively small amounts compared with benzpyrene.

Elimination in altered form. The alkali-soluble fraction of the fluorescent fowl bile to which reference has already been made was acidified with dilute HCl and extracted with ether. The ether extract was dried over Na_2SO_4, and the

solvent evaporated in N_2 under reduced pressure. If the benzene solution of the residue showed a fluorescence in the ultra-violet beam, the fluorescence spectrum of the solution was examined before chromatographic adsorption on alumina. If not, the solution was adsorbed on alumina in an attempt to separate any fluorescing material from the spectrographically interfering colouring matter.

Chromatographic adsorption. On filtering a benzene solution, prepared as described above, through a 5–7 cm. tower of washed alumina, it was found that in almost every case the fluorescent material in the solution was strongly adsorbed forming a fluorescent ring or zone at the top of the column (see Table 2). The initial filtrate was usually non-fluorescent. Some difficulty was found in eluting the fluorescent material from the alumina; in each case, benzene plus MeOH was the best eluant. The eluates were evaporated to dryness in N_2 under reduced pressure, and the residue dissolved in a small quantity of EtOH or benzene, depending on its solubility for fluorescence spectrum analysis. The fluorescence spectra of extracts prepared in this way compared with the fluorescence spectra of the hydrocarbons under examination are also recorded in Table 2. 3:4-Benzphenanthrene and fluoranthene showed only a general fluorescence, and consequently fluorescence spectrum analysis was not suitable for the detection of these compounds.

An examination was made of the alkali-soluble fraction of 200 ml. normal fowl bile, which were extracted by the method described above. On filtration of the benzene solution through a tower of alumina, a small pale-blue fluorescent zone was formed at the top of the column, but the filtrate of the chromatogram was non-fluorescent and the eluate of the small fluorescent zone, while showing a pale-blue fluorescence, did not exhibit a specific fluorescence spectrum.

The fluorescence spectrum examination of the derivatives of the hydro-

Table 2

Hydrocarbon	Fluorescence spectra: Hydrocarbon	Fluorescence spectra: Derivative from bile	Chromatogram
Carcinogenic:			
3:4-Benzphenanthrene	(369–431)	Nil	Nil
3:4-Benzpyrene	(403–408) (409–411) (426–434) (454–459)	(428–446) (458–468)	Greenish yellow zone
1:2:5:6-Dibenzanthracene	(394–399) (404–409) (418–423) (429–438) (444–448)	Non-specific	Green ring
Cholanthrene	(391–406) (411–426) (431–441)	Non-specific	Green ring
Methylcholanthrene	(393–404) (415–429) (444–447)	Non-specific	Tower completely blue
Non-carcinogenic:			
Anthracene	(381–391) (399–414) (424–436) (453–460)	Non-specific	Ill-defined 2–3 cm. zone
Phenanthrene	(374–384) (396–408) (418–431) (446–456)	Nil	Nil
Pyrene	(373–375) (378–380) (383–386) (389–392) (393–397)	(385–389) (392–395) (403–413) (426–436)	Blue zone
2′:6-Dimethyl-1:2-benzanthracene	(386–396) (406–421) (431–441)	(404–421) (429–451)	Blue zone
Fluoranthene	(406–486)	Non-specific	Blue zone

Wave-lengths in Å. $\times 10^{-1}$.

carbons found in the bile showed that the derivatives of pyrene and 2′:6-dimethyl-1:2-benzanthracene, like that of benzpyrene, have a banded fluorescence spectrum, while those of 1:2:5:6-dibenzanthracene, cholanthrene, methylcholanthrene, anthracene, 2′:7-dimethyl-1:2-benzanthracene and fluoranthene have a general fluorescence spectrum. The results of the fluorescence spectrum examination, however, must be regarded as tentative in view of the small quantities of fluorescent material used in the chromatographic adsorption experiments.

Solubility of hydrocarbons in fowl bile and serum. The extraction of unchanged benzpyrene from the bile raised the question of the relative solubilities of benzpyrene and the other hydrocarbons in fowl bile and serum.

A slight excess of the hydrocarbon was mixed with 1 ml. of normal fowl bile and 1 ml. of normal fowl serum and incubated at 37° for at least 24 hr. with occasional shaking. The solubility of the hydrocarbon in the bile or serum was determined by the fluorescence of the solution in the ultraviolet beam. The solutions were examined every few hours, and the incubation was terminated when no further evidence of solution could be detected. It may be noted here that direct visual fluoroscopy is a very convenient method for detecting the more obviously fluorescent hydrocarbons or their derivatives in bile, but that negative findings do not necessarily mean that no fluorescent substance is present, since a weak fluorescence may easily be masked by bile pigments. Finally the ultraviolet fluorescence of samples of the filtered solutions of bile and serum was compared with that of normal samples.

An estimate of the relative solubilities of the hydrocarbons in fowl bile and serum was based on: (*a*) the time taken for the bile or serum to exhibit a distinctive fluorescence due to the hydrocarbon, and (*b*) a visual comparison of the intensity of fluorescence of the saturated solution in the bile or serum after filtration from any undissolved hydrocarbon. The order of solubility in fowl serum from maximum to minimum was: (1) benzpyrene and fluoranthene; (2) 3:4-benzphenanthrene and pyrene; (3) cholanthrene, methylcholanthrene, and 2′:6-dimethyl-1:2-benzanthracene; (4) anthracene. 1:2:5:6-Dibenzanthracene, phenanthrene, 2′:7-dimethyl-1:2-benzanthracene and chrysene, which were also tested, appeared to be insoluble. Similar solubility findings were obtained with rabbit and mouse sera. This order of solubility, however, made no allowance for the relative intensity of fluorescence of solutions of the hydrocarbons of equal concentration, and in order to make allowance for this factor ether extracts of the serum saturated with the hydrocarbon were compared with fluorescent standards of the hydrocarbon in the same solvent. The results indicated that benzpyrene was more soluble than cholanthrene, methylcholanthrene, anthracene and 2′:6-dimethyl-1:2-benzanthracene in fowl serum.

Similar results were also obtained for the solubility of the hydrocarbons in fowl bile. In this case, however, probably owing to the masking effect of the bile pigments, anthracene and 2′:6-dimethyl-1:2-benzanthracene, which were slightly soluble in fowl serum, appeared to be insoluble in the bile. Otherwise, the solubilities of the hydrocarbons in fowl bile and serum were similar.

Discussion

Various carcinogenic and non-carcinogenic hydrocarbons have been compared with benzpyrene as regards their elimination in bile after intravenous injection in the fowl. Part of the injected benzpyrene could be recovered unchanged from fistula bile, the remainder being eliminated as a fluorescent

derivative, or, possibly, further metabolized. So far as could be judged, the amount of benzpyrene injected did not affect the mode of elimination. None of the other hydrocarbons tested was found to be eliminated unchanged in fowl bile. A number of the hydrocarbons, 1:2:5:6-dibenzanthracene, cholanthrene and methylcholanthrene (carcinogenic), and anthracene, pyrene, 2′:6- and 2′:7-dimethyl-1:2-benzanthracene and fluoranthene (non-carcinogenic), after intravenous injection in the fowl, gave rise to the production of fluorescent bile. Subsequent investigation of the fluorescent substances in such samples of bile showed that the fluorescence was not due to the elimination of the unchanged compound but to a derivative of the hydrocarbon analogous to 'BPX'.

Since benzpyrene was the only hydrocarbon eliminated unchanged, it was thought that the solubility of benzpyrene in fowl bile and serum, which appeared to be somewhat greater than that of the other hydrocarbons, might explain this elimination. Visual examination of the fluorescence of the hydrocarbons in fowl, rabbit and mouse sera indicated that benzpyrene was more readily soluble than the other hydrocarbons examined, and this was confirmed by estimations of the concentration of the hydrocarbons in fowl serum. However, Lorenz & Andervont [1936] found that methylcholanthrene and 1:2:5:6-dibenzanthracene have the same solubility in fowl serum, and record differences in the solubility of 1:2:5:6-dibenzanthracene in horse, fowl and dog sera. Also, Brock *et al.* [1938] have observed that benzpyrene is less soluble than 1:2:5:6-dibenzanthracene in horse serum. These results, indicating a variation in the solubility of 1:2:5:6-dibenzanthracene and in the relative solubility of hydrocarbons in sera from different species, suggest that if the partial elimination of the hydrocarbon from the animal body in an unchanged form depends on its solubility in serum, then variable results are to be expected in different species. Although in our experiments 1:2:5:6-dibenzanthracene appeared to be insoluble in fowl serum, the lipins of the serum would presumably dissolve the hydrocarbon to some extent, and variations in the lipin content of the serum may account for differing solubilities. It would also be reasonable to expect the species to affect the solubility and evidence that factors other than lipin content may influence the result is provided by our recent finding that a sample of horse serum, from which the lipin fraction had been removed by ether extraction, appeared to dissolve benzpyrene as readily as a control sample of the normal serum, and a more detailed study of the solvent action of serum is being made.

Dobriner *et al.* [1939] report that 'the absorption bands of unchanged 1:2:5:6-dibenzanthracene were present in the fraction containing neutral compounds of the faeces of injected rats, mice and, rarely, of rabbits'. In our experiments, we did not detect the elimination of 1:2:5:6-dibenzanthracene in an unchanged form in fowl bile, and it is possible that this result may be explained by a difference in the metabolism of this compound in the fowl, or by the fact that we used much smaller quantities of the hydrocarbon.

The elimination of triphenylmethane from the animal body in an unchanged form has been described by Miriam *et al.* [1927]. In a feeding experiment these authors recovered 27·5 % of the hydrocarbon in the urine of rabbits, but only traces in the faeces. In the dog, 35 % of this compound was recovered unchanged from the urine and the faeces were not examined. A much larger fraction of triphenylmethane than of benzpyrene is therefore eliminated unchanged, and the mode of elimination is different, since it takes place chiefly in the urine and not in the faeces. The presence of traces of triphenylmethane in the faeces suggests that the hydrocarbon is partly excreted in the bile but re-absorption may have occurred in the bowel followed by ultimate excretion in the urine.

Considering the elimination of the hydrocarbons in altered form, pyrene and 2′:6-dimethyl-1:2-benzanthracene are excreted in fowl bile as fluorescent derivatives which show a banded fluorescence spectrum in organic solvents and are strongly adsorbed on alumina. In these properties, they resemble benzpyrene. After intravenous injection of the other hydrocarbons (1:2:5:6-dibenzanthracene, cholanthrene, methylcholanthrene, anthracene, 2′:7-dimethyl-1:2-benzanthracene and fluoranthene) benzene solutions of the alkali-soluble fraction of the fluorescent fowl bile were strongly adsorbed on alumina as fluorescent zones. The fluorescence of the bile and the adsorption on alumina constitute presumptive evidence that these hydrocarbons were partly excreted in the bile as fluorescent derivatives, although the solutions when examined either before or after chromatographic adsorption showed a general and not a specific fluorescence spectrum.

Boyland & Levi [1935; 1936] have found that when anthracene is fed to rats and rabbits different stereoisomeric forms of 1:2-dihydroxy-1:2-dihydroanthracene and their glucuronic acid derivatives are excreted in the urine. It is possible that the metabolism of anthracene to this derivative may be responsible for the fluorescence of fowl bile collected after injection of the hydrocarbon. This derivative of anthracene, however, is a neutral compound, and would not be present in the alkali-soluble fraction which was strongly adsorbed on alumina from benzene solution. This suggests the formation of another derivative of anthracene which may be peculiar to the fowl.

Boyland & Levi [1938] and Boyland *et al.* [1941] have also described the isolation of fluorescent hydroxy derivatives from the urine of rabbits fed on a diet containing 1:2:5:6-dibenzanthracene or 3:4:5:6-dibenzcarbazole, and Dobriner *et al.* [1939] have described the isolation of a hydroxy derivative of 1:2:5:6-dibenzanthracene from the 'excreta' of rabbits, rats and mice injected with this compound. 4′:8′-Dihydroxy-1:2:5:6-dibenzanthracene has been synthesized by Cason & Fieser [1940] and is considered by them to be probably identical with the rat and mouse metabolite of this carcinogen, but not with the rabbit metabolite. The metabolism of 1:2:5:6-dibenzanthracene in the fowl to a hydroxy derivative would account for the fluorescence of the bile collected after the intravenous injection of this hydrocarbon, and for the presence of a fluorescent derivative in the alkali-soluble fraction of the bile.

Summary

The elimination after intravenous injection in the fowl of ten polycyclic hydrocarbons (enumerated in Table 1) has been compared with that of 3:4-benzpyrene.

It has previously been shown that 3:4-benzpyrene is eliminated in the bile and faeces, partly unchanged and partly as a fluorescent derivative, 'BPX', which is probably a monohydroxybenzpyrene.

In the present experiments, it was found by analyses of fowl bile that:

(1) Unlike benzpyrene, none of the other hydrocarbons was eliminated unchanged in recognizable amounts.

(2) Hydrocarbons 3, 4, 5, 6, 8, 9, 10 and 11 were eliminated as fluorescent derivatives. Of these, the derivatives of hydrocarbons 8 and 9 showed a characteristic banded fluorescence spectra, analogous to that of 'BPX', while the others showed only a general fluorescence.

(3) Hydrocarbons 1 and 7 gave rise to no fluorescent derivative in the bile.

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143. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

5. THE EFFECT OF MUSCLE EXTRACT AND ALIPHATIC BASES

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Research on the chemotherapy of cancer is still in the stage of examining substances which inhibit tumour growth rather than cure it by causing complete disappearance of the tumour. There have been many reports of substances present in tissues which inhibit both normal and malignant growth. In many cases the effect has been shown on growth in tissue culture; thus Walton [1914] found that the growth of cultures was inhibited by liver extract and many explants of parenchymatous cells were inhibited by muscle extracts. Roffo [1938] also describes the inhibition of tissue growth by muscle extract. Heaton [1926] described the inhibitory action of liver extract and obtained similar but more pronounced effects with certain fractions from yeast. Baker & Carrel [1925] obtained similar inhibition of growth with ether-soluble material from serum, brain, liver, egg and embryo tissue, and Medawar [1937] with malt extracts. Recently Brues *et al.* [1940] have attempted to fractionate the inhibitory factor of liver and showed that some of the activity is due to ethanolamine.

Analogous effects have been obtained in animals. Heaton [1929] observed inhibition of body growth and of tumour growth in animals treated with extracts of yeast or malt. This effect of yeast extracts on malignant growths has been confirmed by Lewisohn *et al.* [1940]. Tumour-inhibitory substances have also been obtained from mammary tissue and placenta [Macfadyen *et al.* 1939], spleen [e.g. Lewisohn, 1938], liver [Dobrovolskaïa-Zavadskaïa & Zephiroff, 1940] and heart muscle [Roffo, 1938]. There is thus a rough parallelism between the actions of these tissue extracts on tissue cultures and tumours growing in animals.

It is possible that tissues contain growth inhibitors and promoters, so that the effect of any extract will depend upon the method of preparation. It should be noticed that factors which inhibit the growth of established tumours do not necessarily inhibit the induction of tumours: thus Watson [1933] found that feeding with liver increased the carcinogenic response in tar-treated mice, while according to Maisin *et al.* [1938] yeast extracts inhibit the production of benzpyrene tumours in mice.

From the point of view of cancer chemotherapy the results obtained by Roffo [1938] with muscle extracts appeared to be most striking, and although the results described below are not so remarkable as those obtained by Roffo, the tumour growth-inhibiting action of muscle extract was confirmed and investigated.

Table 1. *The ratio of growth rate of treated to that of untreated mouse tumours*

In columns A, B, C and D the numerators in the fractions refer to the increase in size (length plus breadth in mm.) of the tumour, while the denominators refer to the number of days required for that growth; each column A, B, C and D refers to an individual mouse. If any diminution in size occurred during the treatment period this is recorded by a minus sign and the inhibition is considered as over 100%.

	Toxicity L.D. 50 for mice (oral unless otherwise stated)	Daily dose	Effect on grafted sarcomata M.C.D.B.1. inhibition %	Effect on spontaneous carcinomata: (i) Growth in control period. (ii) Growth in treatment period. (iii) Ratio ii/i												Average effect	
				A			B			C			D			Inhibition %	Probability (P) that inhibition is due to chance
				(i)	(ii)	(iii)	(i)	(ii)	(iii)	(i)	(ii)	(iii)	(i)	(ii)	(iii)		
Muscle extract M_1 (prepared with cold 5 N HCl)	2·0 ml.	0·5 ml.	20	$\frac{17}{14}$	$\frac{2}{11}$	0·15	$\frac{10}{24}$	$\frac{2}{14}$	0·34	$\frac{8}{8}$	$\frac{2}{14}$	0·14	$\frac{10}{17}$	$\frac{8}{14}$	0·97	60	0·05
Muscle extract M_5 (prepared with cold N H_2SO_4 and $Ca(OH)_2$)	3·0 ml.	0·5 ml.	18	$\frac{10}{14}$	$\frac{-4}{14}$	−0·40	$\frac{10}{10}$	$\frac{3}{14}$	0·22	$\frac{12}{10}$	$\frac{16}{14}$	0·95	$\frac{9}{10}$	$\frac{1}{10}$	0·11	64	0·05
M_5 intraperitoneal	1·0 ml.	0·2 ml.	18	$\frac{15}{14}$	$\frac{-10}{14}$	−0·66	$\frac{11}{18}$	$\frac{-1}{14}$	−0·11	$\frac{9}{13}$	$\frac{4}{14}$	0·41	$\frac{13}{12}$	$\frac{5}{14}$	0·33	101	0·02
Dialysed muscle extract	3·0 ml.	1·0 ml.	6	$\frac{13}{22}$	$\frac{5}{14}$	0·61	$\frac{11}{9}$	$\frac{-5}{14}$	−0·29	$\frac{11}{15}$	$\frac{5}{14}$	0·49	$\frac{16}{24}$	$\frac{2}{14}$	0·21	74	0·02
Muscle extract: pyridine-soluble	2·0 ml.	0·4 ml.	1	$\frac{16}{17}$	$\frac{10}{14}$	0·76	$\frac{11}{16}$	$\frac{2}{14}$	0·21	$\frac{6}{6}$	$\frac{5}{14}$	0·36	$\frac{7}{12}$	$\frac{4}{14}$	0·49	54	0·02
Muscle extract: EtOH-soluble	3·0 ml.	0·4 ml.	25	$\frac{20}{10}$	$\frac{16}{14}$	0·57	$\frac{10}{12}$	$\frac{6}{14}$	0·52	$\frac{16}{12}$	$\frac{5}{14}$	0·27	$\frac{7}{10}$	$\frac{3}{14}$	0·31	48	0·01
Muscle extract: $CCl_3.COOH$-soluble	>3·0 ml.	0·5 ml.	18	$\frac{8}{9}$	$\frac{-2}{10}$	−0·22	$\frac{15}{14}$	$\frac{-2}{14}$	−0·13	$\frac{11}{31}$	$\frac{0}{14}$	0	$\frac{16}{14}$	$\frac{-4}{14}$	−0·25	115	0·01
Muscle extract: insoluble in $CCl_3.COOH$	>3·0 ml.	0·5 ml.	15	$\frac{14}{7}$	$\frac{14}{14}$	0·50	$\frac{10}{8}$	$\frac{11}{14}$	0·63	$\frac{13}{12}$	$\frac{8}{14}$	0·54	$\frac{11}{14}$	$\frac{8}{14}$	0·73	40	0·2
Muscle extract: phosphotungstic acid ppt.	>3·0 ml.	0·5 ml.	21	$\frac{12}{17}$	$\frac{1}{14}$	0·10	$\frac{15}{17}$	$\frac{4}{14}$	0·32	$\frac{10}{23}$	$\frac{4}{14}$	0·66	$\frac{9}{7}$	$\frac{-3}{10}$	−0·23	79	0·02
Muscle extract: phosphotungstic acid-soluble	>3·0 ml.	0·5 ml.	0	$\frac{8}{12}$	$\frac{8}{14}$	0·86	$\frac{10}{8}$	$\frac{10}{10}$	1·25	$\frac{8}{12}$	$\frac{6}{14}$	0·64	$\frac{10}{12}$	$\frac{11}{14}$	0·94	8	0·6

β-Alanine	200 mg.	50 mg.	7	$\frac{8}{14}$	$\frac{2}{14}$	0·25	$\frac{18}{24}$	$\frac{17}{14}$	1·62	$\frac{14}{14}$	$\frac{14}{9}$	1·55	$\frac{11}{18}$	$\frac{5}{14}$	0·58	0	—
Aneurin	—	0·5 mg.	11	$\frac{11}{17}$	$\frac{0}{14}$	0	$\frac{24}{17}$	$\frac{6}{14}$	0·30	$\frac{25}{26}$	$\frac{8}{14}$	0·59	$\frac{8}{24}$	$\frac{2}{14}$	0·43	67	0·1
Arcaine sulphate	50 mg.	5 mg.	11	$\frac{10}{5}$	$\frac{13}{14}$	0·47	$\frac{10}{7}$	$\frac{5}{14}$	0·25	$\frac{10}{6}$	$\frac{0}{14}$	0	$\frac{8}{13}$	$\frac{-2}{11}$	−0·30	89	0·01
Betaine	120 mg.	25 mg.	10	$\frac{23}{11}$	$\frac{10}{14}$	0·34	$\frac{8}{25}$	$\frac{1}{14}$	0·22	$\frac{9}{12}$	$\frac{8}{14}$	0·76	$\frac{7}{14}$	$\frac{7}{14}$	1·00	42	0·1
Carnosine nitrate	—	5 mg.	0	$\frac{13}{17}$	$\frac{0}{10}$	0	$\frac{15}{9}$	$\frac{15}{14}$	0·64	$\frac{13}{8}$	$\frac{8}{11}$	0·45	$\frac{16}{18}$	$\frac{-2}{12}$	−0·19	77	0·05
Choline chloride	120 mg.	25 mg.	9	$\frac{20}{30}$	$\frac{5}{14}$	0·54	$\frac{14}{12}$	$\frac{8}{14}$	0·49	$\frac{13}{11}$	$\frac{-1}{14}$	−0·06	$\frac{10}{12}$	$\frac{0}{14}$	0	75	0·02
Creatine	200 mg.	50 mg.	11	$\frac{14}{11}$	$\frac{-7}{14}$	−0·39	$\frac{11}{10}$	$\frac{11}{14}$	0·71	$\frac{12}{5}$	$\frac{11}{14}$	0·33	$\frac{10}{17}$	$\frac{8}{14}$	0·97	59	0·2
Creatinine	200 mg.	50 mg.	4	$\frac{9}{18}$	$\frac{-3}{14}$	−0·43	$\frac{12}{10}$	$\frac{0}{14}$	0	$\frac{10}{14}$	$\frac{6}{14}$	0·60	$\frac{10}{17}$	$\frac{13}{14}$	1·57	56	0·3
Ethanolamine	40 mg.	10 mg.	9	$\frac{11}{8}$	$\frac{-8}{11}$	−0·53	$\frac{11}{15}$	$\frac{1}{14}$	0·10	$\frac{15}{12}$	$\frac{-4}{14}$	−0·23	$\frac{7}{10}$	$\frac{3}{14}$	0·31	109	0·01
Methylguanidine	12 mg.	3 mg.	16	$\frac{13}{17}$	$\frac{5}{14}$	0·45	$\frac{10}{11}$	$\frac{11}{14}$	0·86	$\frac{12}{14}$	$\frac{4}{14}$	0·33	$\frac{17}{14}$	$\frac{8}{14}$	0·47	47	0·2
Trimethylamine oxide hydrochloride	40 mg.	10 mg.	17	$\frac{13}{7}$	$\frac{9}{14}$	0·35	$\frac{12}{11}$	$\frac{-2}{14}$	−0·13	$\frac{10}{19}$	$\frac{3}{14}$	0·41	$\frac{18}{12}$	$\frac{-2}{14}$	−0·10	87	0·01
Ethylenediamine hydrochloride	100 mg.	10 mg.	4	$\frac{9}{14}$	$\frac{6}{14}$	0·66	$\frac{8}{18}$	$\frac{7}{14}$	1·12	$\frac{7}{25}$	$\frac{2}{14}$	0·51	$\frac{13}{13}$	$\frac{-4}{14}$	−0·28	50	0·1
Putrescine	50 mg.	10 mg.	13	$\frac{13}{9}$	$\frac{7}{11}$	0·43	$\frac{10}{10}$	$\frac{6}{14}$	0·43	$\frac{8}{14}$	$\frac{1}{14}$	0·13	$\frac{10}{18}$	$\frac{8}{14}$	1·03	50	0·1
Cadaverine hydrochloride	100 mg.	25 mg.	4	$\frac{7}{24}$	$\frac{-5}{14}$	−1·22	$\frac{13}{7}$	$\frac{2}{11}$	0·10	$\frac{10}{10}$	$\frac{-3}{10}$	−0·30	$\frac{8}{7}$	$\frac{7}{12}$	0·51	123	0·05
Spermine hydrochloride	60 mg.	5 mg.	8	$\frac{8}{13}$	$\frac{3}{14}$	0·33	$\frac{9}{14}$	$\frac{4}{14}$	0·44	$\frac{12}{14}$	$\frac{-4}{14}$	−0·33	$\frac{8}{11}$	$\frac{-3}{14}$	−0·29	96	0·02
Tetradecamethylene-diamine dihydrochloride	2 mg.	1 mg.	19	$\frac{7}{20}$	$\frac{1}{14}$	0·20	$\frac{9}{21}$	$\frac{-1}{14}$	−0·17	$\frac{13}{9}$	$\frac{2}{14}$	0·10	$\frac{10}{8}$	$\frac{10}{14}$	0·57	86	0·05

Experimental

The methods of testing were the same as those used in the previous paper [Boyland, 1940] and the results are given in Table 1. Muscle extracts were prepared by extracting minced ox heart muscle with 5 volumes of acid for 48 hr. at room temperature. Extract M_1 was prepared with $5N$ HCl and the acid after extraction was neutralized with $NaHCO_3$. Extract M_5 was prepared with N H_2SO_4, the acid neutralized with $Ca(OH)_2$ and the precipitated $CaSO_4$ removed. Both these extracts produced significant inhibition of growth of spontaneous mouse tumours.

A greater effect was obtained when the extract was given by intraperitoneal injection, but as definite effects were obtained with the oral administration of the extracts, the latter method was used in subsequent experiments.

Fractionation of muscle extract. A sample (50 ml.) of the extract M_5 was dialysed against distilled water in a cellophane bag for 24 hr. at 5°; this treatment did not remove all the activity. The precipitate obtained from another sample (100 ml.) on addition of 20 ml. 25 % trichloroacetic acid was suspended in 50 ml. 1 % $NaHCO_3$; the trichloroacetic acid filtrate was boiled for 45 min. to destroy the acid and was made up to 50 ml. The trichloroacetic acid precipitate was not significantly active in inhibiting tumour growth, but the filtrate showed more activity than the original extract. Two preparations of the trichloroacetic acid filtrate were evaporated to dryness on the water bath; the dry residue of one was extracted with absolute alcohol and the other with pyridine. In each case the solvent was removed and the residue dissolved in water equal in volume to that of the trichloroacetic acid used. In both cases the extracts had some action, but not as much as the original trichloroacetic acid filtrate.

Another portion of the neutralized filtrate was extracted with butyl alcohol in a continuous extractor for 48 hr. The extract, which contained purines, was inactive against grafted sarcomata.

Phosphotungstic acid precipitation. Phosphotungstic acid (20 % in 5 % H_2SO_4) was added to 500 ml. of trichloroacetic acid filtrate of extract M_5 containing 5 % H_2SO_4 until no more precipitate was formed. After 24 hr. at 5° the precipitate was removed by centrifuging and washed with a small amount of 5 % H_2SO_4. The centrifugate and washings were combined and treated with a slight excess of $Ba(OH)_2$. The excess $Ba(OH)_2$ was removed with CO_2 and the neutral filtrate concentrated on the water bath to 250 ml. This phosphotungstic acid-soluble material had no tumour growth-inhibiting action. The precipitate was suspended in 50 % aqueous acetone and shaken with excess $Ba(OH)_2$ solution (saturated at 50°). The barium sulphate and phosphotungstate were filtered off and washed twice with water at 50°. The excess of $Ba(OH)_2$ was removed by CO_2 from combined filtrate and washings. The solution of bases was then concentrated to 250 ml. This material precipitated by phosphotungstic acid reduced the growth rate of tumours. This relatively clear-cut separation of the activity present in a trichloroacetic acid extract with phosphotungstic acid indicates that bases either present in muscle or liberated during the prolonged extraction with acid are responsible for the action of the extract.

Aliphatic bases. The extract M_1 was shown to contain choline and probably contained ethanolamine which would arise from the phosphatides during the prolonged extraction. Several bases which might be expected to occur in muscle extract, and related compounds, were tested; of these arcaine sulphate (tetramethylenediguanidine sulphate), carnosine nitrate, choline chloride, ethanolamine, trimethylamine oxide hydrochloride, cadaverine hydrochloride, spermine

hydrochloride and tetradecamethylenediamine dihydrochloride, all had marked inhibitory actions on spontaneous tumours. No experiments on the effects of summation of action with these compounds were carried out, but it is possible that the effects of different compounds administered in a mixture would be summed. It therefore seems probable that the tumour-inhibiting action of material obtained by prolonged acid extraction of heart muscle is due to several bases present rather than to one particular compound. The most effective substances were ethanolamine and cadaverine. Heart muscle contains lecithin and cephalin and the prolonged acid extraction would liberate choline and ethanolamine [cf. Kay, 1931].

Cadaverine is a simple diamine like tetradecamethylenediamine which had previously been tested [Boyland, 1938]. These compounds are similar to the aromatic diamines which have been examined and it is possible that their actions are related. The relatively high activity of ethanolamine and choline suggests that other hydroxy-amines should be examined. *p*-Aminophenol has been tested and is not very effective, but some nitrosophenols such as α-nitroso-β-naphthol are quite effective [Boyland & Boyland, 1939; and unpublished observations]. Other ethanolamine derivatives, diethanolamine and triethanolamine, were similar to ethanolamine in their action.

Hypoxanthine (5 mg. per day), agmatine (25 mg. per day) and inositol (10 mg. per day) were all inactive against the grafted sarcomata M.C.D.B.1. Other substances which were only tested against the grafted sarcoma included an extract of Pecten muscle, which produced 15 % inhibition in growth, glutamine (10 mg. per day, 10 % inhibition), nicotinamide (30 mg. per day, 11 % inhibition) and trigonelline (25 mg. per day, 4 % inhibition).

The aliphatic bases which have been found to inhibit tumour growth are all diamines or hydroxy-amines. The active compounds can therefore be expressed as P-X-P^1, where P and P^1 are polar groups. Similar activity has been found [Boyland, 1940] with aliphatic dicarboxylic acids such as malonic acid, and these compounds can be represented by the same general formula where P and P^1 are carboxyl groups. In the case of β-alanine, where P is NH_2 and P^1 is a COOH group, there is no activity. A large number of aromatic bases have been tested, and in this series also maximum activity was obtained with compounds containing two terminal amino groups, which can be represented by the same general formula.

Summary

By prolonged cold acid extraction of heart muscle a preparation is obtained which inhibits the growth of grafted sarcomata and spontaneous carcinomata in mice.

The active material is heat-stable, is not precipitated by trichloroacetic acid, but is precipitated by phosphotungstic acid. The activity appears to be due to many of the bases present rather than to any one compound.

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